

METHOD FOR EXAMINING REACTIVITY
AND METHOD FOR DETECTING A COMPLEX

5 This application is a division of U.S. Patent Application
No. 09/942,662, filed August 31, 2001, now abandoned, which is
incorporated herein by reference.

BACKGROUND OF THE INVENTION

Field of the Invention

10 The present invention is directed to examining multiple
specimens at a time for multiple items, and provides a method
in which matrix substrates with biological samples having
different properties and origins bound thereto are prepared,
and on each matrix region, oligonucleotides having different
15 sequences, proteins or drugs are spotted in an array, whereby
multiple specimens are examined at a time for multiple items.

 The present invention also relates to a method in which,
by using an oligonucleotide having a known base sequence as a
detection probe to detect whether a complex is formed by
20 intermolecular bond with this oligonucleotide, detection is
made as to whether or not components having a capability of
bonding to the above-described detection probe are contained,
and to a detection substrate having the oligonucleotide as a
detection probe fixed on its surface, which is used
25 exclusively for this detecting method.

Related Background Art

 In identification of partial sequences included in the
base sequence of a nucleic acid molecule, detection of a
target nucleic acid contained in a sample originated from an
30 organism or identification of genus or species for various
bacteria based on the characteristics of the gene DNA of the

5 bacteria, a procedure may be used in which two or more probe
DNAs having known base sequences are used to detect whether or
not the nucleic acid molecule is a nucleic acid molecule
specifically binding to each probe DNA, namely making
hybridization with each probe DNA. As an effective approach
10 to performing speedily and accurately examination of the two
or more probe DNAs by the hybridization method, a procedure is
proposed in which a probe array of two or more probe DNAs
arranged regularly on a solid phase is used to detect at a
time whether or not the nucleic acid molecule is a nucleic
15 acid molecule specifically binding to each probe DNA.

Among common methods for producing such probe arrays, as
described in European Patent No. 373203 (EP 0373203 B1) for
example, methods are known in which predetermined nucleic acid
probes are synthesized in an array form on a solid phase, and
20 methods in which a plurality of nucleic acid probes
synthesized in advance is supplied in an array form on the
solid phase.

Prior technical documents disclosing the former methods
include, for example, U.S. Patent No. 5,405,783. Also, as one
25 example of the latter methods, a method in which cDNAs are
arranged in an array form on a solid phase using
micropipetting is disclosed in, for example, U.S. Patent No.
5,601,980 and "Science", Vol. 270, pp. 467, (1995).

The probe array that is prepared using these methods may
30 be an array in which nucleic acid probes are arranged on a
solid phase at a high density of 10000 or more probes per
square inch. A hybridization reaction with multiple probes is
carried out at a time by dipping this high-density probe array
into a specimen solution, and in so doing, the base sequence
35 of genes is analyzed based on the base sequence of nucleic
acids that hybridize. This method has an advantage in that

5 probes are arranged at a high density on a substrate with a small area, thereby making it possible to conduct a multiple-item examination at the same time with a small amount of samples to reduce the burden associated with sampling from the subject.

10 As a method of preparing the high-density probe array for the above-described application on the substrate by the DNA synthesis process, a method in which a photolithography technology is applied is disclosed in the aforesaid U.S. Patent No. 5,405,783, but highly advanced equipment is
15 required for implementing this method, and the method is not easy enough for anyone to use.

Also, in the case where the number of specimens is large but the number of required examination items is not so large, the integration degree of DNA probes on the probe array
20 corresponding to the number of examination items does not need to be very high. Rather, there may be cases where it is necessary to prepare a large number of probe arrays with a small number of desired DNA probes fixed, using a simpler method.

25 Actually, in the field of clinical examination, there are not necessarily many cases where examinations for more than 10000 items are required. For example, in the case of group health examination and the like, there may be cases where it is more important to examine a large number of specimens with
30 a limited number of items. For examining a large number of specimens in this way, a system is required such that presence of diseases can be speedily examined through comparison with standard samples with respect to each specimen.

In addition, the amount of a DNA specimen is generally
35 small as compared to that of an oligonucleotide capable of being synthesized and used in the probe. For using it in a

5 normal form in which the probe array substrate is dipped into
the specimen solution for a hybridization reaction, the amount
of specimen DNA allowing the substrate to be dipped
sufficiently is required. Therefore, the size of the DNA
probe array substrate is limited depending on the amount of
10 specimen DNA, and thus the array needs to be highly dense.
Alternatively, as a result of diluting the specimen solution
to ensure its volume for the size of the probe array
substrate, the concentration of DNA in the specimen solution
is reduced, and a procedure is adopted for prolonging the
15 reaction time to compensate for the reduced concentration.

Also, since the amount of sampled specimens is limited
inherently because the specimen is an extract from tissues,
and because it is subjected to pre-processing for making a
specimen solution for use in the hybridization reaction,
20 specifically, extraction of nucleic acid, single-strand
formation thereof, and process for labeling, the amount of
finally obtained samples is very small. In order to
compensate for that, the sample is subjected to processing for
amplification of the amount of DNA, such as amplification
25 processing by PCR reaction, before it is used for examination
and studies. However, there exists a disadvantage in that
because separately prepared primers are required for carrying
out a PCR reaction, such processing can be applied only to
specific genes of which primer sequence is known. In
30 addition, there exist sequences that can easily be amplified
and sequences that can hardly be amplified in the process of
PCR reaction, and thus, the efficiency of reaction (rate of
amplification) is not uniform. For example, in the case where
the content of a specific mRNA in the total amount of
35 extracted mRNA is examined to determine diseases or like based
on the content, standard samples providing criteria should be

5 always prepared to make correction on the above-described amplification rate.

Although the amount of the specimen solution required for a hybridization reaction decreases as the size of the substrate is reduced, there is a limitation on downsizing of the substrate in association with handling. Specifically, it is possible in principle to enhance array density and reduce the number of probes to be placed on the array to downsize the substrate, but if an extremely small substrate is used, a dedicated handling apparatus is required in the process of processing such as hybridization reaction and detection thereafter, which cannot be practical.

Also, for examining cDNA for mRNA that is transcribed with reflection of the process of development of a certain organism, cDNA for mRNA that is transcribed with reflection of each phase in the process of culturing a certain cell, cDNA for mRNA that is transcribed by interaction with drugs, and so on, a DNA array with multiple types of test samples arranged is used. Examples of arraying this test sample are described, for example, in the above-described "Science", Vol. 270, pp 467, (1995). In this case, test samples arrayed on the substrate are dipped using as a probe solution the labeled DNA of known sequence that is derived from genes having a specific function, whereby a hybridization reaction is carried out.

If a plurality of items is to be examined at a time using this methodology, DNA probes labeled with different types of fluorescent reagents (fluorochromes) should be prepared depending on the number of items. When a detection is made, those different types of fluorescent reagents (fluorochromes) must be distinguished from one another, and therefore, their wavelengths and the like should be different as a matter of course. Of course, detection filters corresponding to

5 respective fluorescent reagents (fluorochromes) are also
needed for a detector.

 This need for a simultaneous examination of multiple
items for multiple specimens is not characteristic exclusively
of a hybridization reaction among genes (DNA).

10 For example, it is also important to examine multiple
items with a small amount of samples as to the interaction
between genes and other substances, such as interaction
between genes and proteins (DNA binding proteins) and
screening of chemicals that are bound to genes. Detection of
15 former DNA binding proteins is used to elucidate the control
mechanism of gene expression by proteins, such as
transcription accelerators. However, in the present
situation, methods in which DNA fragments are bound to
proteins, and thereafter complexes are analyzed by gel
20 electrophoresis, are adopted. In this method, the number of
specimens that can be analyzed at a time is limited due to the
usage of gel electrophoresis, and considerable time is
required for analysis.

 For the field of drug development, there may be cases
25 where an examination of interaction between genes and
administered drugs constitutes an important item in the
progress of research, but it takes relatively a large amount
of time and effort to obtain chemically synthesized products
for use in drugs to be researched, and it can be considered
30 that a reduction in the amount of drugs to be used in
screening results is a significant improvement in efficiency
of their research.

 As introduced above, there are cases where when a complex
is formed using an interaction between two substances, such as
35 hybridization between DNAs, formation of a complex of DNA and
a protein, and interaction of a drug compound with gene DNA,

5 or the presence or absence of the interaction causing a
complex to be formed, is examined, the amount of samples of
one of those two substances is limited, and the limited amount
of samples should be used to conduct a series of desired
examinations across multiple types as to the presence or
10 absence of formed complexes. That is, development of an
examination method in which consumption of samples required
for individual examinations can be reduced to carry out the
examination across multiple types more efficiently within a
limited amount of samples is desired.

15 SUMMARY OF THE INVENTION

An object of the first invention is to provide a method
of examining multiple specimens at a time for multiple items,
for example a method in which matrix substrates with
biological samples having different properties and origins
20 bound thereto are prepared, and on each matrix region,
oligonucleotides or proteins having different sequences and
drugs are spotted in an array form, whereby multiple specimens
are examined at a time for multiple items.

Another object of the invention is to provide a method in
25 which multiple specimens can also be examined at a time for
multiple items in a similar way for interaction between
chemicals, especially drugs, and cDNA, binding of proteins to
cDNA and the like.

An object of the second invention is to provide a new
30 method in which an oligonucleotide with a known base sequence
and which can be obtained relatively easily is used as a
detection probe. When, for a limited amount of sampled
specimens, the presence or absence of a bonding capability to
the above-described oligonucleotide as a detection probe or
35 the degree of the bonding capability is examined by the

5 presence or absence of complexes formed between those two
substances, or efficiency thereof is evaluated, consumption of
specimens required for evaluation for each type of
oligonucleotide as a detection probe can be reduced. In
addition, the invention also has an object to provide a
10 detection substrate with the above-described oligonucleotide
being fixed as a detection probe in a predetermined region of
its surface, which is used exclusively for the method, and to
provide a method of preparing the detection substrate.

The examination method of the first invention capable of
15 achieving the above-described objects is a method in which a
reactivity between a first sample and a plurality of second
samples having different properties from one another is
examined at a time,

characterized in that in a defined region on a substrate
20 with the first sample bound on the entire surface in advance,
the second samples are placed independently of one another as
spots having a smaller size than the above-described defined
region, and then the reactivity between the above-described
first sample and each of the second samples is tested.

25 The matrix of biological samples related to the invention
that is usefully used for the above examination method is
characterized in that two or more types of biological samples
of different origins exist in respective matrix regions
separated on the substrate.

30 According to the invention, a substrate with biological
samples having different properties and origins (e.g. nucleic
acids and proteins) bound in a matrix form in advance can be
provided.

There is also provided a method in which DNA probes like
35 oligonucleotides, cDNAs, proteins or chemicals are spotted in
an array form on the above-described substrate with biological

5 samples having different properties and origins placed in a
matrix form to carry out the reaction, and the presence or
absence of another sample bound to a certain biological
sample, the degree of the bonding, and the presence or absence
of interaction is quickly examined for multiple items at the
10 same time.

In this method, the area occupied by one specimen is very
small, because two or more types of specimens are placed on
one substrate. Therefore, there is an advantage in that the
amount of required cDNA may be very small as compared to the
15 case where the hybridization reaction is carried out using a
conventional DNA array with an enormously large number of DNA
probes bound in an array form in advance. Also, there is
neither a limitation on the size of the DNA array substrate
nor an inconvenience in handling.

20 Also, by providing a method in which examination can be
carried out even with a small amount of samples, the method
opens the door to areas in which examination could not be
carried out, because conventionally, a sufficient amount of
samples cannot be obtained, for example, a new examination
25 area in which mRNA obtained from tissues is directly examined.

In addition, according to the invention, chemicals,
proteins and nucleic acids can be examined at the same time
under the same reaction condition on the same substrate.

A method of detecting object components in test samples
30 according to the second aspect of the invention is a method in
which using as a detection probe oligonucleotide with a known
base sequence, complexes formed between the above-described
oligonucleotide and the object components are detected to
examine whether or not the object components capable of
35 binding to the above-described oligonucleotide are contained

5 in the liquid test samples, or evaluate the degree of binding capability thereof,

characterized in that there is at least one type of the above-described oligonucleotide used as a detection probe with a known base sequence,

10 there are at least two types of test samples to be examined, and

a detection substrate with the above-described one or more types of oligonucleotide for detection probes bound to predetermined sections respectively on a predetermined solid
15 substrate is used.

The above-described method comprises steps of:

spotting a plurality of predetermined amounts of sample solution for each spot so that a predetermined array shape is formed in the spotted position, for each of the above-
20 described two or more types of test samples, in each section with the oligonucleotide to detect probes bound in advance;

detecting the presence or absence of complexes formed between the above-described oligonucleotide and the object component, for the above-described plurality of spots for each
25 test sample, respectively; and

determining whether or not the object component capable of binding to the above-described oligonucleotide is contained, or the degree of the capability of binding, based on the result of the above-described detection.

30 Also, the present invention provides a detection substrate that is exclusively used when the above-described method of the invention is carried out. That is, the detection substrate of the present invention is a detection substrate with two or more oligonucleotides having known base
35 sequences different from one another fixed on a solid substrate, characterized in that:

5 the above-described plurality of oligonucleotides are
bound and fixed in predetermined sections, respectively, so
that one type of oligonucleotide exists in each section, and
a plurality of the above-described sections in which
oligonucleotides are fixed is placed in a matrix form on the
10 surface of the above-described solid substrate.

The method of preparing the detection substrate of the
present invention is a method suitable for preparation of the
above-described detection substrate of the invention, and
specifically, is a method of preparing a detection substrate
15 with two or more oligonucleotides having known base sequences
different from one another fixed on a solid substrate,
characterized in that:

for the above-described solid substrate, a substrate with
a plurality of sections separated in a matrix form in advance
20 formed on the surface thereof is used,

the above-described plurality of oligonucleotides is
supplied into predetermined sections in predetermined amounts
using printing by an ink jet process, respectively, so that
one oligonucleotide is present in each section, and

25 the supplied oligonucleotides are fixed in the
predetermined sections.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows one example of an arrangement aspect of
defined regions on a substrate in the present invention;

30 FIGS. 2A and 2B show one example of matrices in the
present invention, wherein FIG. 2A is a plan view, and FIG. 2B
is a 2B-2B sectional view thereof;

FIG. 3 is a schematic explanatory view of a specimen
solution discharging method by a bubble jet process that is an
35 embodiment of the present invention;

5 FIG. 4 is a sectional view of a bubble jet head 105 taken in the 4-4 line in FIG. 3;

 FIG. 5 shows a layout of 64 discharged DNA probes on each black matrix;

 FIG. 6 shows one example of detection substrates of the present invention, illustrating schematically a situation in which sections in which oligonucleotides being detection probes are fixed are arranged in a matrix form, and a plurality of cDNAs are spotted in a two-dimensional array form onto each section as detection samples;

15 FIG. 7 illustrates schematically arrangements of respective probes in the detection substrate with 64 DNA probes bound to sections arranged in the form of a 8 x 8 matrix, respectively;

 FIG. 8 shows schematically a pattern of a spot 64 x 64 array in which 64 test samples are spotted in the form of a two-dimensional 8 x 8 array on each section, for the detection substrate on which sections with probes fixed therein are arranged in the form of the 8 x 8 matrix;

25 FIG. 9 shows schematically a result of spotting 64 test samples in the form of the two-dimensional 8 x 8 array on each section for 64 probes fixed in sections arranged in the form of the 8 x 8 matrix to carry out the hybridization reaction; and

 FIG. 10 shows an example of the structure of sections delimited by hydrophobic frame-structured walls provided on the detection substrate of the present invention, and arranged in the form of the 8 x 8 matrix.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

 One embodiment of the present invention will be described below referring to FIG. 1. FIG. 1 shows a substrate surface

5 with 64 defined regions formed thereon, wherein each region
(matrix) measures 1 mm by 1 mm, and a space x between regions
can be selected freely. For methods of preparing biological
sample binding matrix substrates, for example, a method can be
used in which the solution of a first sample (e.g. biological
10 sample) is printed on the entire surface of defined regions on
the substrate as a "solid print pattern" by coating and ink
jet processes, or is supplied by methods, such as chemical
synthesis on the substrate, and is bound in a matrix form on
the substrate through adsorption to the substrate or chemical
15 reaction between functional groups existing in the biological
sample and functional groups existing on the substrate.
Furthermore, the situation in which the first sample is bound
on the entire surface of defined regions means a situation in
which the first sample is bound across the entire surface,
20 such that when a second sample and samples thereafter are
supplied in these defined regions, these reactions occur
without being limited to the positions in the above-described
regions in which the samples are supplied. For example, the
first sample may be fixed in layered form on the entire
25 surface, or the masses of molecules constituting the first
sample may be dispersed on the entire surface in high density
with micro-spaces being kept among them.

The defined regions on this substrate may previously be
provided on the substrate as a well constituted by sections
30 separated in a pattern formed by walls of hydrophobic
compounds.

Also, when using a substrate with a nucleic acid (cDNA)
being a biological sample fixed thereon as the first sample,
two or more probe DNAs possibly included in cDNA are contacted
35 with cDNA on the substrate as the second sample and samples
thereafter. Products of the reaction with the above-described

5 probes are detected on the above-described solid phase to
detect the presence or absence of probe DNA sequences in the
above-described cDNA, two or more probes are supplied in an
array form as mutually independent spots in each matrix with
various kinds of cDNA bound in the defined regions, thereby
10 making it possible to perform simultaneous detection with two
or more probes.

Also, on the nucleic acid (cDNA) matrix, two or more
types of chemicals or proteins that are possibly bound to cDNA
are contacted with the probe DNA on the substrate as mutually
15 independent spots, thereby making it possible to perform a
multiple-item examination composed of these reactions at the
same time. Multiple-item screening of DNA binding proteins
and DNA binding chemicals can be performed at the same time by
detecting the presence of binding of chemicals or proteins to
20 probes on the solid phase.

The present invention is characterized by supplying probe
DNA, proteins and chemicals in a form of droplets of small
amounts on the matrix on which biological samples, such as
cDNA are applied, wherein different types of samples are
25 arranged in an array form, thereby making it possible to
perform simultaneous multiple-item processing.

Combinations of the first sample fixed in advance on the
substrate and the second sample and sample thereafter that are
reacted with the first sample may include the following
30 combinations.

Specific examples of the matrix or the like formed of
defined regions on the substrate for use in the present
invention will be described below.

Shapes of Matrices with Biological Samples Bound Thereto

35 The shapes of matrix patterns are not particularly
limited, but linear, square and rectangular shapes are

5 preferred in that they can be treated irrespective of how specimens are supplied. Of course, shapes such as circles and ellipses will cause no problems.

Materials that are fixed to the substrate as a first sample may include unknown base sequences derived from
10 organisms, cDNA libraries, mRNA libraries, sets of two or more DNA and RNA, known DNA and RNA synthesized or derived from organisms or sets thereof, chips of cloned oncogenes, protein fractions including at least one type of protein derived from organisms, proteins of single type, mixtures of known proteins
15 of different types, and chemicals.

Density of Matrices with Bound Biological Samples

The density of matrices is not particularly limited, but for a preferred form, the density of 400 per centimeter square is preferable. For this preferred density of 400/cm², the
20 size of one matrix is a 500 μ m square in the case of a square shape. If samples to be arranged as spots on the array are 100 μ m in diameter, 25 spots are arranged in total, 5 spots high by 5 spots wide. Also, if the diameter of sample solution is 20 μ m, the number of spots that can be arranged in
25 a row is 25, and 625 spots can be arranged in total.

Preparation of a Substrate with Biological Samples Bound Thereto

Samples originated from organisms (biological samples) include nucleic acids and proteins. Nucleic acids include,
30 for example, mRNA and cDNA, and methods for binding them on the substrate include a method in which a nucleic acid extracted and purified in advance is applied to the substrate to fix the nucleic acid by adsorption and an electrostatic bond, and a method in which the nucleic acid is fixed by
35 providing a covalent bond through a chemical reaction with

5 functional groups on the substrate using amino groups of the nucleic acid.

The method using negative electric charges of DNA is a method in which the nucleic acid is electrostatically bound to a solid carrier subjected to a surface treatment with positive
10 polymeric ions, such as polylysine, polyethyleneimine and polyalkylamine, and then blocking of excessive positive ions is carried out, which is generally used.

Types of Functional Groups of Solid Phases and Nucleic Acids

15 Combinations of functional groups that are used for fixation include, for example, a combination of epoxy groups (on solid phase) and amino groups (amino groups in nucleic acid probe terminals or base groups). Methods for introducing epoxy groups to the solid surface include, for example, a
20 method in which polyglycidyl methacrylate having epoxy groups is applied to the solid surface composed of resin, and a method in which a silane coupling agent having epoxy groups is applied to the solid surface made of glass and is reacted with glass.

25 Binding of Proteins to the Solid Phase

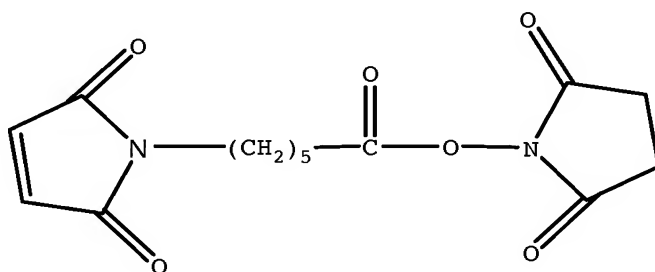
Methods of binding proteins to the substrate include methods using adsorption, as in the case of nucleic acid, and methods using electrostatic binding. Furthermore, methods of forming a covalent bond include those using SH groups of
30 cysteine residues in addition to the above-described methods using amino groups.

Methods of Fixing Proteins Using Thiol Groups

Methods using cysteine residues for fixing proteins include, for example, methods using combinations of maleimide
35 groups and thiol groups (-SH). That is, the treatment is performed so that the solid surface has maleimide groups,

5 whereby thiol groups of cysteine residues supplied to the solid surface can be reacted with maleimide groups of the solid surface to fix proteins.

Various kinds of methods may be used to introduce maleimide groups to the solid surface. This can be achieved
10 by, for example, reacting an aminosilane coupling agent with a glass substrate, and then reacting its amino groups with a reagent containing N-(6-maleimidocaproyloxy) succinimide) expressed by the following structural formula (EMCS reagent: manufactured by Dojin Co., Ltd.)



15

As another example, a reagent containing succinimidyl 4-(maleimidophenyl) butyrate can be used to react with, preferably amino groups.

DNA Matrix Structures Composed of Hydrophobic Matrices

20 As an additional form of fixing biological samples, a method can be used in which a well composed of, for example, hydrophilic and hydrophobic matrices is formed on the solid surface, a structure to prevent coupling among spots is provided in advance, and the DNA prove is supplied in the well
25 to carry out a coupling reaction.

Materials of Matrices/Wells

When a prove solution is put on the separated matrix to carry out the coupling reaction, it is preferable that portions constituting the well are hydrophilic, and portions
30 corresponding to the wall surface of the well and the partition between the well and a neighboring well are composed

5 of materials whose surfaces are less compatible with the probe solution. Due to such a treatment, the probe solution can be smoothly supplied to a desired well even if some positional deviation occurs when the probe solution is supplied to the well.

10 One example of a matrix in this embodiment is shown in FIGS. 2A and 2B. FIG. 2A is a plan view, and FIG. 2B is a 2B-2B sectional view thereof. This matrix has a structure in which a matrix pattern 125 having a frame structure with formed recesses 127 (wells) placed in the form of a solid
15 phase 103 is provided. The wells 127 separated from one another by the matrix 125 (height) are provided as through-holes (cut-off portions) in the matrix pattern. The side of the matrix pattern is constituted by height, and the bottom 129 has the exposed surface of the solid phase 103. The
20 portion of the exposed surface of the solid phase 103 forms a surface that can be coupled to the probe, and the probe is fixed in a predetermined recess.

Materials forming the matrix pattern include, for example, metals (chrome, aluminum, gold, etc.) and resins.
25 They include resins, such as acryl, polycarbonate, polystyrene, polyimide, acrylate monomers and urethane acrylate, and photosensitive resins, such as photoresists having black dyes and black pigments contained therein. For specific examples of photosensitive resins, UV resists, DEEP-
30 UV resists, ultraviolet cured resins and the like can be used. UV resists may include negative resists, such as cyclized polyisoprene-aromatic bisazide resists, phenol resin-aromatic azide compound resists, and positive resists, such as novolac resin-diazonaphthoquinone resists.

35 DEEP-UV resists may include, for example, radiation dispersion type polymer resists, such as polymethyl

5 methacrylate, polymethylene sulfone, polyhexafluorobutyl
methacrylate, polymethyl isopropenyl ketone and bromo poly 1-
trimethylcylilpropine, and dissolution inhibiting resists,
such as cholate o-nitrobenzyl ester as positive type resists,
and may include polyvinylphenol-3-3'-diazidediphenylsulfone,
10 and polymethacrylate glycidyl as negative type resists.

Ultraviolet cured resins may include polyester acrylate,
epoxy acrylate and urethane diacrylate containing
approximately 2 to 10% by weight of one or more types of
photopolymerization initiators, which are selected from
15 benzophenone and substituted derivatives thereof, oxime
compounds, such as benzyl, and so on.

For curbing reflection by the material forming the matrix
during detection, light-blocking materials can be effectively
used to form the matrix pattern. For this purpose, it is
20 effective to add black pigments in the above-described resin.
Examples of black pigments that can be used are carbon black
and black organic pigments.

Here, if the matrix 125 is composed of resin, the surface
of the matrix 125 is hydrophobic. This structure is preferred
25 when an aqueous solution is used as a solution containing
probes to be supplied to the well. That is, even if the prove
solution is supplied to the well, the prove solution is
supplied to a desired well quite smoothly. Also, if different
probes are supplied among adjacent wells at the same time,
30 intermingling (cross-contamination) of different probe
solutions supplied among these wells can be prevented.

The thickness of the matrix (height from the solid
surface) is determined in light of the matrix pattern forming
process and the volume of the well, but it is preferably in
35 the range of 1 to 20 μm . Particularly, it can be considered
as a thickness range that effectively prevents cross-

5 contamination when the probe solution is supplied to each well through an ink jet process.

Types of Samples to be Spotted

Samples to be spotted as droplets onto the above-described matrices of biological samples include probe nucleic
10 acids, proteins and chemicals, such as drugs.

For probe nucleic acids, in addition to deoxyribonucleic acid, any type of nucleic acid, such as ribonucleic acid and peptide nucleic acid, may be used as long as it has nucleic acid bases. The length of the oligonucleotide probe is not
15 particularly limited, but it is preferably in the range of 10 mer to 50 mer for carrying out an accurate hybridization reaction with cDNA.

For proteins, their own fluorescence can be used to detect DNA bonding proteins.

20 Some chemicals can also be detected with their own fluorescence.

Method of Preparing Sample Arrays

Methods of spotting sample solution on defined positions in the size of several tens to several hundreds of microns
25 include a pin system, an ink jet system and a capillary system.

The pin system refers to a method in which the sample is attached to the pin tip, for example, in such a manner that the pin tip is contacted with the surface of the solution
30 including the sample, and then the tip is mechanically contacted with the solid phase, thereby preparing a sample array. The capillary system using a capillary is such that the sample solution once sucked up to the capillary is mechanically contacted with the solid phase through the tip of
35 the capillary as in the case of the pin system, thereby supplying the sample solution in an array form. For these

5 spotting operations, various commercially available
apparatuses from various companies may be used. These methods
are considered as most preferable in the sense that any sample
DNA can be supplied. However, as for quantification, the
problem may be in that viscosity varies depending on the
10 length and concentration of DNA. For proteins, these methods
are also preferred in the sense that they are deposited
independently of the size and viscosity of molecules, but not
suitable for quantitative analysis.

15 Outline of Sample Array Preparing Methods Through the Ink Jet Process

Samples capable of being discharged in an ink jet process
include chemicals in addition to nucleic acids and proteins.

In the ink jet process, because a shearing force is
exerted, the length of dischargeable nucleic acids and the
20 size of dischargeable proteins are often limited. However,
this process is superior in quantification to the pin system
and capillary system, and is used more suitably than other
systems with respect to the discharge of chemicals.

Dischargeable nucleic acids are limited to those with a
25 relative length to bases of 5 kb or smaller, and dischargeable
proteins are limited to those of 1000 K daltons or less. As
for chemicals, any chemicals can be discharged.

Any liquid can be used for discharging and supplying
samples with ink jets, as long as this liquid is capable of
30 being discharged from ink jets. The above-described liquid
discharged from the head is shot to a predetermined position.
When being mixed with nucleic acid probes and during
discharge, the above-described nucleic acid probes are not
damaged.

35 In terms of dischargeability from the ink jet,
particularly from the bubble jet head, with respect to the

5 properties of the above-described liquid, it is preferable
that its viscosity be in the range of 1 to 15 cps and its
surface tension be 30 dyn/cm or larger. Also, if the
viscosity is in the range of 1 to 5 cps and the surface
tension is in the range of 30 to 50 dyn/cm, the positions in
10 which the liquid is spotted on the solid phase are extremely
accurate.

Therefore, if the stability of the nucleic acid during
discharge or the like is taken into consideration, a nucleic
acid probe of, for example, 2 to 5000 mer, particularly 2 to
15 1000 mer, is preferably contained in the solution at a
concentration of 0.05 to 500 μ M, particularly 2 to 50 μ M.

FIG. 3 is a schematic explanatory view of a specimen
solution discharging method through the bubble jet process,
which is one embodiment of the present invention. In FIG. 3,
20 reference numeral 101 denotes a liquid supplying system
(nozzle) retaining a solution, including a specimen, as a
discharge liquid in such a manner that the solution is capable
of being discharged, reference numeral 103 denotes a solid
phase having a nucleic probe bound thereto with which the
25 above-described specimen is reacted, and reference numeral 105
denotes a bubble jet head that supplies heat energy to the
above-described liquid to discharge it, and is a type of ink
jet head. Reference numeral 104 denotes a liquid including
the specimen discharged from the bubble jet head. FIG. 4 is a
30 4-4 line sectional view of the bubble jet head 105 in FIG. 3.
In FIG. 4, reference numeral 105 denotes the bubble jet head.
Reference numeral 107 denotes a liquid including a specimen
solution to be discharged. Reference numeral 117 denotes a
substrate portion having a heat generation portion to provide
35 discharge energy to the above-described liquid. The substrate
portion 117 includes a protective layer 109 formed from

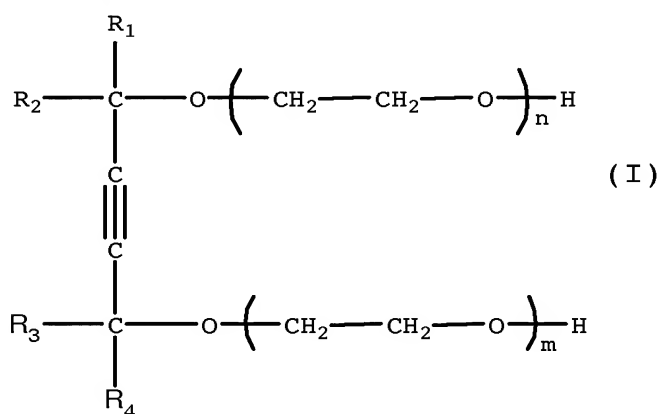
5 silicon oxide and the like, electrodes 111-1 and 111-2 formed from aluminum and the like, an exothermic resistor layer 113 formed from nichrome and the like, a heat storage layer 115, and a support 116 formed from aluminum having a good heat-release property.

10 The liquid 107, including the specimen, comes to a discharge orifice (discharge outlet) 119, and forms a meniscus 121 with a predetermined pressure. Here, when electric signals are applied to the electrodes 111-1 and 111-2, a region (foaming region) denoted by reference numeral 123
15 abruptly releases heat, and the liquid 117 contacted therewith is discharged and flies toward the solid surface 103. The amount of the liquid that can be discharged using a bubble jet head having such a structure varies depending on the size of its nozzle, but can be controlled approximately to 4 to 50
20 picoliters, which is extremely useful as a means for placing specimen probes at a high density.

In terms of dischargeability from the ink jet, particularly from the bubble jet head, with respect to the properties of the above-described liquid, it is preferable
25 that its viscosity be in the range of 1 to 15 cps and its surface tension be 30 dyn/cm or larger. Also, if the viscosity is in the range of 1 to 5 cps and the surface tension is in the range of 30 to 50 dyn/cm, the positions in which the liquid is spotted on the solid phase are extremely
30 accurate.

Therefore, if the stability of nucleic acid during discharge or the like is taken into consideration, a nucleic acid of, for example, 2 to 5000 mer, particularly 2 to 1000 mer, is preferably contained in the solution at a
35 concentration of 0.05 to 500 μ M, particularly 2 to 50 μ M.

5 The composition of a discharged liquid is not particularly limited, as long as the liquid has no substantial influence on the nucleic acid probe when it is mixed with the nucleic acid probe and when it is discharged from the ink jet, and it can be normally discharged to the solid phase using the
10 ink jet. Preferable liquids include glycerin, urea, thiodiglycol or ethylene glycol, isopropyl alcohol, and acetyl alcohol expressed by the following formula:



15 In the above formula (I), R_1 , R_2 , R_3 and R_4 represent alkyl groups, specifically linear or branched alkyl groups having 1 to 4 carbon atoms, m and n represent integer numbers, respectively, wherein m and n equal 0, or $1 \leq m + n \leq 30$, and if $m + n = 1$, m or n equals 0.

20 More specifically, a liquid containing 5 to 10% by weight (wt%) of urea, 5 to 10 wt% of glycerin, 5 to 10 wt% of thiodiglycol, and 0.02 to 5 wt%, more preferably 0.5 to 1 wt%, of acetylene alcohol presented by the above formula (I) may be suitably used.

25 The detecting method of the present invention is a method of detecting a complex formed between an oligonucleotide for detection probes and an object component, which is used for the purpose of making an evaluation/examination as to whether

5 or not a component capable of binding to the oligonucleotide
and forming a complex in a liquid test sample for use as a
detection probe whose base sequence is known, and as to the
degree of binding capability thereof if such a component
exists in the sample. For detecting this complex, the
10 oligonucleotide for detection probes is fixed in advance on
the solid surface substrate, whereby this fixed
oligonucleotide is bound to the object component contained in
the test sample, and the formed complex is separated while it
is fixed on the solid substrate. On the basis of a
15 methodology for detecting complexes using proper detecting
means, the amount of test samples required at this time is
reduced to a very low level. Also, the detection accuracy and
sensitivity are kept at a sufficiently high level.

That is, in this methodology providing a base for the
20 present invention, since surface density of the
oligonucleotide for detection probes that is fixed on the
solid surface substrate can be kept at a predetermined value,
the amount of the formed complex is proportional to the
binding capability of the object component, and is also
25 proportional to the concentration of the object component
contained in the test sample that is contacted with the solid
surface substrate and is made to act on the oligonucleotide.
Taking advantage of this characteristic, the test sample is
contacted only with the surface, with the oligonucleotide for
30 detection probes actually fixed thereon. The contact area is
limited to a certain degree, whereby the amount of the used
test sample is also limited to a certain degree.
Specifically, by adopting a means for spotting a predetermined
minimal amount of liquid in the form of droplets, the contact
35 area and the amount of liquid put thereon are controlled with
good reproducibility. The amount of the complex that would be

5 automatically fixed on the solid surface substrate with
formation is detected for this limited contact area, thereby
achieving detection accuracy and sensitivity that are
essentially as high as those in the case of dipping the entire
solid substrate with oligonucleotide for detection probes
10 fixed thereon in the liquid test sample.

The complex is detected by the label bound on the surface
of the substrate. When a complex of the oligonucleotide and
the labeled test sample is formed and the individual spots are
sufficiently spaced from each other, detection can be carried
15 out independently for each spot. Therefore, if given or
larger spaces are provided between adjacent spots, even though
there are spots for different test samples nearby, only spots
for desired test samples can be selected to continue detection
work without being influenced by those spots. In the
20 detection method of the present invention, in order to satisfy
reliably, the requirement that given or larger spaces be
provided between adjacent spots, a defined array is formed in
the spot position as a result of providing predetermined
spaces as spaces between spots, and a predetermined amount of
25 sample solution is spotted for each spot to make the spot area
(contact area) constant, or make the spot diameter constant to
ensure reproducibility, because the shape of the spot (contact
surface) is generally a circle. As a matter of course, for
precluding the influence of adjacent spots, a space between
30 spots is selected such that optical signals (fluorescent) and
the like derived from the adjacent spots are not mixed in the
detection system, in the light of the measured area (diameter
in the measurement range) of the detection system selected as
appropriate in accordance with the spot diameter. Also, as a
35 matter of course, the detecting method of the present
invention really shows its advantages in the case where there

5 exist two or more types of test samples, and they are detected simultaneously.

On the other hand, on the surface to which a plurality of spots of such array forms are provided, one type of oligonucleotide for detection probes should be fixed at a
10 uniform surface density. Also, for the section in which the oligonucleotide for detection probes is fixed, its area and shape are selected as appropriate in accordance with the above-described array space and the total number of spots to be included in a series of arrays. It is also possible to
15 provide sections having different oligonucleotides fixed therein in different regions on the detection substrate to be used, and to place a plurality of sections with two or more oligonucleotides fixed therein, respectively. That is, it can be said that the detecting method of the present invention
20 becomes a more suitable method if used when two or more types of nucleotides are used as detection probes to carry out a series of evaluations simultaneously for a plurality of test samples, with respect to two or more types of object components corresponding to respective oligonucleotides.

25 Generally, in such an evaluation, it is often the case that the oligonucleotides for detection probes are predetermined while only an approximate number of test samples to be evaluated is determined. In such a case, it is preferable that as a detection substrate with oligonucleotides
30 for detection probes fixed thereon in advance, a detection substrate with two or more types of detection probes put thereon systematically, having on the substrate surface in a matrix form sections in which respective oligonucleotides are fixed. In this detection substrate with fixed sections
35 arranged thereon in a matrix form, the unit of a total number of spots that are made in an array form in each section is

5 fixed, but a plurality of these units can be used to carry out the evaluation depending on the number of test samples to be actually evaluated, thus enhancing convenience in practice. Furthermore, for each section arranged in a matrix form, a pattern formed by hydrophobic compounds is preferably provided
10 in its substrate to provide a form in which mutual regions are separated from one another.

In the detecting method of the present invention, nucleic acid molecules may be selected as object components to apply the same when evaluating whether or not they are engaged in a
15 double-strand formation in hybrid substances formed through a hybridization reaction with the oligonucleotide for detection probes. In this case, the method used is an effective method in which an evaluation is made at the same time even for multiple test samples as to whether or not nucleic acid
20 molecules, including base sequences complementary to known base sequences of the oligonucleotide for detection probes, are contained in the test sample. Alternatively, if two or more types of nucleotides for detection probes are provided, and one type of nucleic acid molecules is contained in each
25 test sample, an evaluation can be made for the nucleic acid molecule with the still unknown base sequences as to whether or not the nucleic acid molecule includes base sequences complementary to known base sequences of each oligonucleotide, which is effective, for example, as a means for searching for
30 a gene group having a set of homologies.

The detection substrate of the present invention is a DNA probe substrate with oligonucleotides for use in probes respectively bound to sections arranged in a matrix form in advance. Particularly for the substrate, the bottoms of
35 sections separated by wells (walls) of the frame structure matrix patterns formed in advance by hydrophobic compounds are

5 formed as hydrophilic surfaces, thereby making the binding of
oligonucleotide easier. Also, by providing this hydrophobic
wall, intermingling of DNA probes among adjacent sections can
be curbed more reliably.

Also, using these DNA probe substrates, the test sample
10 is spotted in an array form on the matrix of the
oligonucleotide to carry out the hybridization reaction,
thereby providing a means for checking quickly whether or not
nucleic acid molecules with complementary sequences are
included in each test sample for a certain oligonucleotide
15 probe.

In this method, since the number of test samples that are
used in the hybridization reaction is determined solely based
on the number of spots, the size of the detection substrate is
not limited. By using a substrate with a large area, the
20 section in which each probe is fixed can be widened, and the
necessity to increase density can be eliminated. Thus, since
the section in which each probe is fixed can be increased, a
wide range of methods can be used, such as methods in which a
liquid containing probes is applied to, or printed as, a
25 "solid printed pattern" through an ink jet process on defined
regions on the substrate, or methods in which chemical
synthesis is carried on the substrate, for means for binding
of the probe/oligonucleotide on the substrate.

Also, considering that the probe/oligonucleotide is less
30 expensive and easier to obtain than the test sample, no
significant problem arises even if the area of the region to
which oligonucleotide is bound is more or less increased. In
this case, with respect to various kinds of test samples to be
spotted, it is not necessary to always spot them at a high
35 density. Furthermore, when the test sample is spotted in
small amounts, the concentration of the object component that

5 is contained in the test sample is increased, whereby the
hybridization reaction can be accelerated, making it possible
to perform a highly sensitive detection for a short time. In
addition, application of the detecting method of the present
invention will open the door to fields that could not be
10 considered previously, because a sufficient amount of samples
could not be obtained, for example a new field in which mRNA
obtained from tissues is directly examined.

Furthermore, information of reactivity in association
with the obtained hybridization reaction is analyzed/evaluated
15 in terms of existence/non-existence of complementarity to
various kinds of oligonucleotides/probes, with respect to
nucleic acid molecules contained in a specific test sample,
thereby making it possible to carry out a detection that also
functions similar to the conventional DNA arrays
20 (hybridization reaction with multiple probes for one
specimen).

Furthermore, the detecting method of the present
invention provides a means for evaluating as object components
the interaction between chemicals, in particular drugs and
25 oligonucleotides, the bonding of proteins to oligonucleotides
and the like. Therefore, it can also be used as a means for
examining object components included in the test sample for
multiple items, with respect to a large number of test
samples. In addition, it provides a means for carrying out an
30 examination on the same substrate at the same time and under
the same conditions even for object components of different
properties, such as chemicals, proteins and nucleic acids.

The detecting method of the present invention and the
detection substrate for use exclusively therein will be
35 described further in detail below.

5 FIG. 6 shows an example of applying the detecting method
of the present invention to an embodiment in which cDNA is
used as an object component. A hybrid substance is formed
through a hybridization reaction with an oligonucleotide with
known base sequence that is used for detection probes. In the
10 detection substrate shown in FIG. 6, a plurality of
rectangular sections separated systematically in a matrix form
in advance are provided on the solid surface substrate in a
rectangular form. The rectangular sections are each spatially
isolated by matrix compartments that are surrounding walls.
15 DNA probes that are used for hybridization/probes are each
bound uniformly to the bottom surface of the rectangular
sections.

Also, attached is an enlarged view showing schematically
a situation in which a plurality of test samples including
20 cDNA as an object component, for example two or more types of
cDNA solutions prepared based on m-RNAs collected respectively
are spotted in the form of the two-dimensional array like a
square matrix, in a section with the DNA prove fixed therein.
The detection substrate, the detection probe, components to be
25 detected and the like that are used in the detecting method of
the present invention will be described further in detail.

Oligonucleotides that are Used for Detection Probes

In the detecting method of the present invention, a
deoxyribonucleic acid can be used for an oligonucleotide that
30 is used for detection probes. In addition thereto, a
ribonucleic acid, a peptide nucleic acid and the like can be
used. Types thereof are not limited as long as they have
desired base sequences, are capable of being bound to other
molecules in those portions, and can be fixed on a solid
35 substrate. Also, for portions excluding nucleic acid chains,
those modified with non-nucleic acid atom groups and those

5 having additional structures and the like can also be used as long as the above-described requirements are satisfied.

Furthermore, for this oligonucleotide that is used for detection probes, a desired amount thereof should be artificially prepared or collected, and its base sequence
10 itself should be known. However, its nucleic acid part should have at least two bases. Its base length is not limited in principle, but if the length exceeds that of 100 bases, it becomes significantly more difficult to use as its base length increases when fixing on the solid substrate is to be carried
15 out. Therefore, the base length is preferably restricted to that of 100 bases or less.

For example, when this oligonucleotide is subjected to a hybridization reaction with, for example, nucleic acid molecules that are more than 100 bases long, the length of the
20 oligonucleotide is preferably at least 10 mer for obtaining sufficient bonding. On the other hand, if the length exceeds 50 mer, it is difficult to set conditions for controlling the detection of mismatching, thus making it difficult to select and detect only those that are fully matched. Thus, in order
25 to detect mutations, the length is preferably 60 mer or less.

Furthermore, the range of 10 mer to 60 mer is a preferable range even when the oligonucleotide having desired base sequences, for example DNA is prepared through chemical synthesis.

30 Shapes of Sections with Oligonucleotide Fixed Therein and Arranged in a Matrix Form

The shape of a section itself in which the oligonucleotide for detection probes is bound and fixed is not particularly limited. However, if a test sample is spotted in
35 an array form on this section, generally a simpler shape rather than a complicated outside shape is preferably

5 selected. In addition, when the oligonucleotide is bound and fixed, generally, a simpler shape is preferably selected for providing a uniform surface density in such a section, in view of working efficiency and convenience. Specifically, rectangular forms, for example, line forms, squares and
10 rectangles, are preferably adopted. Of course, in principle, forms whose perimeters are formed by curves, such as circles and ellipses, do not cause any problems.

On the other hand, in the detection substrate of the present invention, when two or more oligonucleotides that are
15 used for detection probes are put on one substrate, sections in which they are fixed are preferably arranged in a matrix form, in view of working efficiency and convenience. Also, preferably, the form and area of each section is unified.

Density of Sections Arranged in a Matrix Form

20 The density of sections arranged in a matrix form is selected as appropriate depending on the number of oligonucleotides that are put on the detection substrate at a time. Moreover, the density of 400 per centimeter square or less is preferable. If the density is $400/\text{cm}^2$, and the form
25 of each section is a square, the size of each section is a $500\text{ }\mu\text{m}$ square. If test samples are closely arranged in an array form as spots with diameters of $100\text{ }\mu\text{m}$, 25 spots are arranged in total with 5 spots high by 5 spots wide. Also, if the diameter of the spot is $20\text{ }\mu\text{m}$, the number of spots that can be
30 arranged in a row is 25, leading to 625 spots in total. Since the detecting method of the present invention has more significant advantages when there is a large number of test samples and they are examined at the same time, the final object of the invention will be more satisfactorily achieved
35 if the density of the section that is arranged is selected so that at least the upper limit of the number of test samples

5 that can be spotted approximately equals the above-described value.

For example, when the detecting method of the present invention is applied to test samples including cDNA, the number of test samples to be examined, specifically the total
10 number of types of cDNA often is approximately as many as 3600. In this case, if the diameter of the spot is 100 μm , the size of one section approximately equals a 6 mm square when 60 spots are arranged in rows and columns, respectively. Also, even if the diameter of the spot is 20 μm , the size of
15 one section should be a 1.2 mm square. In this way, in the detection substrate for use in the detecting method of the present invention, there are not a few cases of application objects where the density of sections that are arranged in a matrix form is preferably selected as 400 per centimeter
20 square or less.

Furthermore, in the detecting method of the present invention, the test sample is spotted as droplets, and in the case where the diameter of the spot is 100 μm , for example, the amount of liquid required for the droplet of one spot is
25 about 25 picoliters. Even if the number of probes for use in examination is selected as 400 (for example, the number of sections of the matrix to be provided on the substrate is 400) for this spot size, the total amount of liquid required for the whole spots may be no more than 10 nanoliters for each
30 test sample, thus making it possible to carry out an objective examination with a minimal amount of liquid.

Also, in the conventional method, in which the detection substrate is dipped in the solution of the test samples, the amount of the required liquid depends on the size of the
35 substrate. Thus, if the amount of the test sample is essentially very small, the size of the substrate should be

5 reduced in accordance with the amount of liquid, and it is
essential to highly integrate probes that are fixed on the
substrate. On the other hand, in the detecting method of the
present invention, the size of the substrate itself can be
freely selected without allowing for the liquid amount of the
10 test sample. In addition, when the oligonucleotide that is
used for detection probes is fixed, the surface density should
be uniform as a matter of course. However, it is not
necessary to highly integrate a plurality of probes to fix
them, making the fixing operation easier.

15 Fixing of Oligonucleotide on the Substrate

As means for fixing the oligonucleotide that is used for
detection probes on the surface of the substrate, a method in
which the oligonucleotide separately prepared in advance is
supplied in predetermined sections by coating or printing to
20 bind the oligonucleotide, or a method in which each
oligonucleotide, specifically, a DNA probe or the like is
synthesized in a solid phase on the substrate to prepare
originally bound DNA, can be used. Furthermore, even in the
case where the oligonucleotide is not DNA, but, for example,
25 is ribonucleic acid or peptide nucleic acid, synthesis on the
substrate can be carried out to bind the oligonucleotide as
described below.

On the other hand, when the oligonucleotide, specifically
DNA or ribonucleic acid, peptide nucleic acid or the like,
30 separately synthesized or collected in advance, is used for
detection probes, a process of fixing the oligonucleotide by
covalent bonding or by electrostatic coupling on the surface
of the substrate can be used.

Synthesis of Oligonucleotide on the Substrate

35 Synthesis of DNA on the substrate includes synthesis on
the silicon substrate using photolithography as a methodology

5 disclosed in U.S. Patent No. 5,445,934. This patent discloses
a method in which high density DNA probe arrays are prepared
by dividing the surface of the silicon substrate into very
small areas and synthesizing DNA for probes. On the other
hand, in the detection substrate for use in the detecting
10 method of the present invention, for example, the size of the
section in which each probe is fixed may be a 0.5 mm square or
larger. Thus, it is not always necessary to enhance the
density. However, also in the detection substrate of the
present invention, photodecomposable protective groups,
15 protective groups that are decomposed by chemicals and the
like, are bound to the nucleic acid in advance, and processes
of masking, light exposure and reaction are repeated, whereby
DNA chains can be synthesized on each section using the
methodology described in U.S. Patent No. 5,445,934 in which
20 four types of nucleic acid bases are bound for each base to
stretch the DNA chain having desired base sequences.

Fixing Oligonucleotide Synthesized or Collected in Advance

As means for carrying out the fixing using electrostatic
25 coupling, a method in which polylysine, polyethyleneimine and
polyalkylamineaone on the solid surface substrate are
subjected to blocking using the negative charge of DNA is
generally used.

However, in the case of the oligonucleotide with a base
30 length of 60 or less, which is not sufficiently long, the
electric charge of its phosphate groups is also weak. Thus,
the binding with the substrate by the above-described method
is not necessarily strong. For the oligonucleotide with a
base length that is not sufficiently long, if a method in
35 which an oligonucleotide with functional groups for a covalent
bond introduced in the terminal of nucleic acid is synthesized

5 in advance, the substrate is subjected to surface processing
suitable for functional groups, and the above-described
functional groups are used to accomplish a covalent bond are
used, stronger binding can be achieved, which is more
preferable.

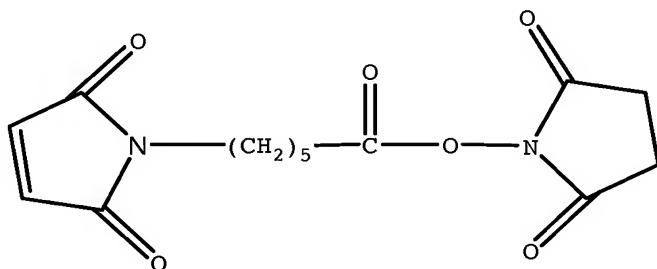
10 Also, in the case where the oligonucleotide is RNA, the
above-described method, which is used for DNA, may be applied.
Alternatively, in the case where the oligonucleotide is a
peptide nucleic acid, its nucleic acid part may be used to
apply the above-described method, which is used for DNA.

15 Types of Functional Groups for Use in Fixing by Covalent
Bond Between the Solid Substrate and Oligonucleotide

When the oligonucleotide is fixed on the solid surface
substrate through a covalent bond, functional groups are
generally introduced in oligonucleotide and the solid surface
20 substrate, respectively, in advance to carry out the reaction
therebetween. For this combination of functions, a preferable
example is a combination in which maleimide groups are
introduced in the surface of the substrate and thiol groups (-
SH) are introduced in the oligonucleotide. Specifically,
25 thiol groups (-SH) are bound to the terminal of the
oligonucleotide while the solid surface is subjected to
processing of forming a coating having maleimide groups. When
the oligonucleotide is supplied to the solid surface, the
thiol groups (-SH) are made to act on and react with the
30 maleimide groups to perform the fixing through the formation
of a covalent bond.

For introducing maleimide groups in the solid surface,
various kinds of methods may be used. For example, an
aminosilane coupling agent is reacted with a glass substrate,
35 and then a reagent (EMCS reagent: manufactured by Dojin Co.,
Ltd.), including N-(6- maleimidocaproyloxy) succinimide

5 expressed by the following formula, whereby a coating layer having maleimide groups can be formed.



As another example, a reagent containing succinimidyl 4-(maleimidophenyl)butyrate can be used to react with amino groups, preferably.

Also, for example, an oligonucleotide with thiol groups introduced therein can be synthesized by using 5' -Thiol-Modifier C6 (manufactured by Glen Research Co., Ltd.) as a five prime-end reagent when DNA is synthesized using a DNA automatic synthesizing apparatus. Furthermore, after synthesis, purification processing by high speed liquid chromatography is applied after a normal deprotection reaction.

Combinations of functional groups capable of being used for fixing by the covalent bond include, for example, a combination of epoxy groups (on the solid surface) and amino groups (the terminal of oligonucleotide) in addition to the above-described combination of thiol groups and maleimide groups. Methods for introducing epoxy groups in the solid surface include, for example, a method in which a coating is applied to the solid surface constituted by polyglycidyl methacrylate having epoxy groups and a method in which a silane coupling agent having epoxy groups is applied to the solid surface made of glass and is reacted with glass.

Supply of Oligonucleotide Solution by the Ink Jet Process

5 There is no particular limitation on the means for
supplying predetermined sections on the surface of the solid
substrate with a solution containing the oligonucleotide to be
fixed thereon, as long as a uniform amount of liquid is
supplied for each unit area. In the case where printing by
10 the ink jet process and the like is used, a "solid print
pattern" is prepared. Then, using an ink jet type printer
head that is used for ink jet printers, the cartridge for the
ink is filled with an oligonucleotide solution instead of the
ink, and printing for a defined area is carried out. If the
15 amount of the liquid to be supplied is small, items with a
large volume, such as an ink cartridge, are not used.
Instead, a structure in which a sample supplying portion, such
as a tube, is connected to a head to supply the
oligonucleotide solution to the head may be used.

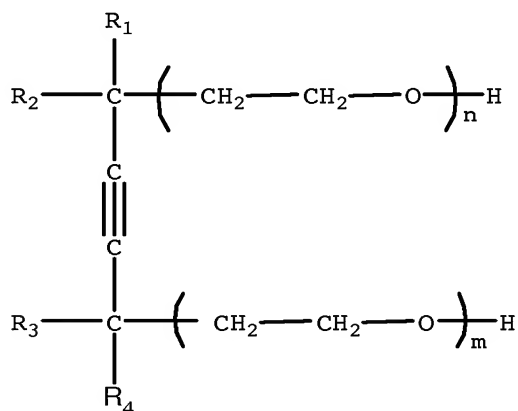
20 For the oligonucleotide solution for the discharge, which
is used in this method, a solution that is capable of being
discharged in the form of ink jets and has a viscosity
suitable for a minimal amount of droplets discharged from the
head to be shot onto a desired position is used. In addition,
25 a solvent to be used is selected from solvents that satisfy
the above-described requirements and do not damage the desired
oligonucleotide in the state of being mixed with the desired
oligonucleotide and during discharge.

 Specifically, in terms of dischargeability from the ink
30 jet head, particularly from the bubble jet head, it is
preferable that, for example, the viscosity is in the range of
1 to 15 cps, and the surface tension is 30 dyn/cm or larger,
as the properties of the solution. In particular, when the
viscosity in the range of 1 to 5 cps and the surface tension
35 in the range of 30 to 50 dyn/cm are selected, the position to
which the solution is shot onto the substrate is extremely

5 accurate, and a supplying method using the bubble jet head is particularly suitably used.

In addition, in terms of the stability of the oligonucleotide during discharge and the like, the supplying means of the ink jet system is further preferred when, for
10 example, a solution containing the oligonucleotide of 2 to 100 mer, particularly 2 to 60 mer, at concentrations ranging from 0.05 to 500 μM , preferably from 2 to 50 μM , is used.

In applying a discharging method of the ink jet system, the liquid composition of the oligonucleotide solution is not particularly limited, as long as the solution practically does
15 not damage the desired oligonucleotide in the state of being mixed with the desired oligonucleotide and during discharge, as described above, and it can be discharged to the surface of the solid substrate using the ink jet. Furthermore, a
20 preferable solution contains, for example, glycerin, urea, thiodiglycol or ethylene glycol, isopropyl alcohol, or acetylene alcohol expressed by the following formula in addition to desired oligonucleotide:



25 In the above formula, R^1 , R^2 , R^3 and R^4 represent alkyl groups, for example linear or branched alkyl groups having 1 to 4 carbon atoms, respectively, and m and n represent 0 or positive integer numbers, respectively, and satisfy $1 \leq m +$

5 $n \leq 30$. In addition, specifically, the liquid composition,
including 5 to 10 wt% of urea, 5 to 10 wt% of glycerin, 5 to
10 wt% of thioglycol, and 0.02 to 5 wt%, more preferably 0.5
to 1 wt% of acetylene alcohol expressed by the formula (I),
allows the discharging method of the ink jet system to be
10 suitably used.

Structure of Matrices Composed of Hydrophobic Walls and Hydrophilic Wells

Also, for sections of the matrix that are provided on the
solid surface, for example, sections of the matrix with
15 hydrophobic walls (barriers) surrounding hydrophilic wells
(recesses) may be formed to prevent coupling between adjacent
sections. A structure may also be used in which the
oligonucleotide solution is supplied to the hydrophilic wells
(recesses) surrounded by the hydrophobic walls (barriers), and
20 the oligonucleotide is fixed only in the bottom of the
hydrophilic wells (recesses).

Materials of Walls/Wells

When the sections are arranged in a matrix form, the
solution of oligonucleotide is supplied to the bottom of the
25 wells (recesses) separated by wall (barrier) patterns to carry
out the binding reaction. It is desirable that the bottom of
the wells (recesses) is wetted densely with the solution, but
the walls (barriers) have poor wettability with the solution.
For example, it is preferable that the solid material
30 constituting the surface of the bottom of the wells (recesses)
is more hydrophilic, and the surface of the walls (barriers)
and the portion corresponding to partitions with neighboring
sections are less hydrophilic. The oligonucleotide solution
supplied in the bottom of the well (recess) is spread across
35 the bottom, but is prevented from finding its way over the
wall (barrier) into adjacent sections. Also, even the droplet

5 erroneously supplied at the position related to the wall (barrier) quickly moves into a desired well (recess) having good wettability. As a result, a predetermined amount of the oligonucleotide solution can be supplied in the well (recess) more reliably.

10 An example of sections arranged in a matrix form that is provided on the detection substrate of the present invention is shown in FIG. 10. The sections in a square matrix form have a structure in which heights (walls) having frame structures are provided on the surface of the solid substrate, and arranged rectangular recesses (wells) are separated. Specifically, the recesses (wells) separated from one another by the heights (walls) having frame structures are formed by coating the entire surface of the solid substrate with a material forming heights (walls), and thereafter providing rectangular through-holes (cut-off portions) to open recesses (wells). Thus, the bottom of the recess (well) has an exposed surface of the solid substrate. The exposed portion of the surface of the solid substrate is subjected to processing for providing a surface to which the oligonucleotide can be bound. As a result, the oligonucleotide is fixed only in the bottom of this recess (well).

Materials forming heights (walls) having frame structures include, for example, metals (chrome, aluminum, gold, etc.) and resins. Resins include, for example, acryl, polycarbonate, polystyrene, polyimide, acrylate monomers and urethane acrylate, and photosensitive resins, such as photoresists, having black dyes and black pigments contained therein. Furthermore, for specific examples of photosensitive resins, UV resists, DEEP-UV resists, ultraviolet cured resins and the like can be used. UV resists may include negative resists, such as cyclized polyisoprene-aromatic bisazide

5 resists, phenol resin-aromatic azide compound resists, and positive resists, such as novolac resin-diazonaphthoquinone resists.

DEEP-UV resists may include, for example, radiation dispersion type polymer resists, such as polymethyl
10 methacrylate, polymethylene sulfone, polyhexafluorobutyl methacrylate, polymethyl isopropenyl ketone and bromo poly 1-trimethylcylilpropine, and dissolution inhibiting resists, such as cholate o-nitrobenzyl ester as positive type resists, and may include borovinylphenol-3-3'- diazidediphenylsulfone,
15 and polymethacrylate glycidyl as negative type resists.

Ultraviolet cured resins may include polyester acrylate, epoxy acrylate and urethane diacrylate containing approximately 2 to 10% by weight of one or more types of photopolymerization initiators, which are selected from
20 benzophenone and substituted derivatives thereof, oxime compounds, such as benzyl, and so on.

When detection is carried out using a fluorescent mark, a light-blocking material can be used effectively for curbing a light reflex by the material forming this height (wall) having
25 a frame structure. For providing a light-blocking property, it is effective to add black pigments in the above-described resins. In this case, black pigments that can be used may include carbon black and black organic pigments.

Furthermore, if the height (wall) having a frame
30 structure is formed by the above-described hydrophobic resin, the surface of the height (wall) is hydrophobic. The configuration in which heights (walls) having frame structures that are formed by hydrophobic materials are provided is more preferable when an aqueous solution is used as a solution
35 containing the oligonucleotide to be supplied to the surface of the substrate of recesses (wells). Even if the aqueous

5 solution is supplied in a position related to the surface of
the height (wall), it is not persistently attached to the
surface of the wall, but gradually moves to the bottom of the
recess (well) located in a lower position. Also, solutions of
different oligonucleotides are supplied to adjacent recesses
10 (wells), but they are separated from each other by the
hydrophobic height (wall). Therefore, intermingling (cross-
contamination) between solutions due to the penetration of the
liquid is prevented.

Furthermore, for the thickness (height from the solid
15 surface) of the height (wall) having a frame structure, the
volume of the recess (well) is selected in view of the amount
of the oligonucleotide solution that is supplied to the recess
(well), and the thickness is determined as appropriate so that
the volume is filled with the solution. Also, depending on
20 methods of forming the height (wall), the thickness is
preferably selected such that it is in the range of 1 to 20 μm
and satisfies the above-described requirement. The thickness
of the height (wall) selected in this way is in the range of
the thickness allowing to effectively prevent cross-
25 contamination between adjacent wells when the oligonucleotide
solution is supplied to each well by the ink jet process.

Types of Specimens

Object components contained in the test sample to which
the detecting method of the invention can be applied include
30 mRNA, cDNA, proteins, cell extracts and chemicals, such as
drugs.

Furthermore, when cDNA is used as an object component, it
is possible to use double-strand cDNA directly. However the
single-strand cDNA marked in advance is preferable in forming
35 hybrid substances efficiently and performing detection thereof
conveniently.

5 On the other hand, mRNA is essentially single stranded,
and it is marked in some way to form marked mRNA, thereby
making it possible to form hybrid substances efficiently and
to perform detection thereof. Furthermore, the amount of mRNA
in the test sample is generally small, and it is an object
10 component more remarkably reflecting the advantage that the
amount of sample solution required for detection can be
reduced to a low level, which is characteristic of the
detecting method of the present invention. However, since RAN
decomposition enzymes tend to admix during handling, a
15 predetermined amount of a substance to prevent decomposition
of mRNA, such as RNA decomposition enzyme inhibitors, such as
diethyl pyrocarbonate, is desirable added in the test sample
solution. In addition to mRNA, similarly, the genome of RNA
viruses can be an object component. In addition, tRNA,
20 ribosomal RNA and the like can be object components.

On the other hand, when the protein is used as an object
component, formed complexes can be detected using the
fluorescence emitted by the protein itself.

Also, some chemicals emit their own fluorescence,
25 enabling formed complexes to be detected using the
fluorescence. Chemicals that do not emit fluorescence may be
marked by methods using functional groups of compounds. Those
to which the detecting method of the invention can be applied
may include, for example, chemicals that can be bound to
30 single-stranded DNA. In addition, they may include, for
example, chemicals that can be bound to single-stranded RNA.

Means for Spotting Test Samples in an Array Form

In the detecting method of the present invention, the
test sample is spotted in an array form in a defined position
35 on the detection substrate. For the purpose of reducing the
amount of the required liquid to a minimal level, the spot

5 diameter is selected so that it is in the range of several
tens to 100 μm . However, with such a spot diameter, the
liquid should be spotted in high uniformity of spotted amounts
and high positional accuracy. As a means for satisfying this
requirement, spotting apparatuses of pin systems, ink jet
10 systems and capillary systems may be used.

The pin system refers to a method in which the test
sample is attached to the pin tip, and the end point thereof
is mechanically contacted with the solid surface, thereby
taking out a fixed amount of the test sample. The capillary
15 system using capillaries refers to a method in which the test
sample solution is sucked up to the capillary on a temporary
basis, and the tip of the capillary is mechanically contacted
with the solid surface as in the case of the pin system,
thereby taking out a fixed amount of the test sample. Various
20 kinds of spotting apparatuses adopting these two systems are
commercially available. Thus, commercially available
apparatuses may be used.

The spotting apparatuses of the pin system and capillary
system enable any types of test samples to be spotted, and are
25 considered as the most preferable methods for unknown test
samples. For example, however, the viscosity of the test
sample solution is varied depending on the length and the
concentration of DNA contained in the test sample. Therefore,
the amount of the spotted liquid varies. Thus, a problem
30 arises in terms of quantification. Also, with respect to
proteins, the viscosity of the test sample solution varies
depending on the size of the molecules and the concentration,
thus raising a problem in terms of quantification.

Spots in an Array Form of Test Samples by the Ink Jet
35 Process

5 Specimens that can be discharged by the ink jet process include chemicals in addition to nucleic acids and proteins.

 In the ink jet process, because a shearing force is exerted, the length of nucleic acids and the size of proteins that can be discharged are limited. However, it is superior
10 in quantification to the pin system and capillary system, and is used more suitably than other systems, particularly with respect to the discharge of chemicals. Preferably, dischargeable nucleic acids are those with a relative length to bases of 5 kb or smaller, and dischargeable proteins are
15 those of 1000 K daltons or less. As for chemicals, their molecular weights are generally small enough compared to nucleic acids and proteins. Therefore, any chemical can be discharged, except for polymers having extremely large molecular weights.

20 FIG. 3 illustrates schematically a method of discharging specimen solution by the ink jet process, particularly the bubble jet process, which is one means that is used for spotting test sample solution in the present invention. In FIG. 3, reference numeral 101 denotes a liquid supply system
25 (nozzle) retaining a solution including a specimen as a discharge liquid in such a manner that the solution is capable of being discharged, reference numeral 103 denotes a solid phase having a nucleic probe bound thereto with which the specimen is reacted, and reference numeral 105 denotes a
30 bubble jet head for providing heat energy to the liquid to discharge it, which is a type of ink jet head. Reference numeral 104 denotes a liquid (droplet) including the specimen discharged from the bubble jet head. FIG. 4 is a sectional view of the bubble jet head 105 described in FIG. 3. In FIG.
35 4, reference numeral 107 denotes a liquid including a specimen solution to be discharged from the bubble jet head 105, and

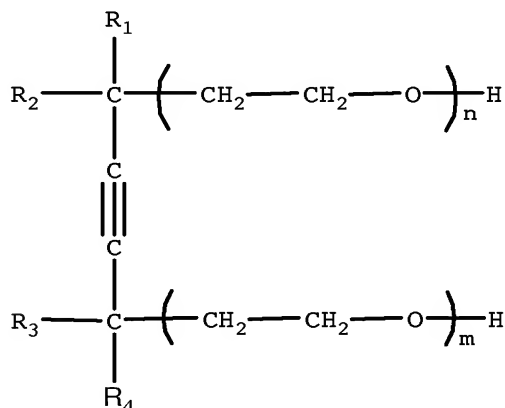
5 reference numeral 118 denotes a substrate portion having a
heat generation portion to provide discharge energy to the
above-described liquid. The substrate portion 118 includes a
protective layer 109 formed by silicon oxide and the like,
electrodes 111-1 and 111-2 formed by aluminum and the like, an
10 exothermic resistor layer 113 formed by nichrome and the like,
a heat storage layer 115, and a support 116 formed by aluminum
having good heat-release properties. The liquid 107 including
the specimen comes to a discharge orifice (discharge outlet)
119 and forms a meniscus 121 with a predetermined pressure.
15 In this situation, when electrical signals are applied to the
electrodes 111-1 and 111-2, a region (foaming region) denoted
by reference numeral 123 abruptly releases heat, and the
liquid 117 contacted therewith is discharged and flies toward
the solid surface 103. The amount of liquid that can be
20 discharged using a bubble jet head having such a structure
varies depending on the size of its nozzle, but can be
controlled to approximately 4 to 50 picoliters, which is
extremely useful as a means for arranging probes at a high
density in a matrix form on the surface of the substrate.

25 In terms of dischargeability from the ink jet,
particularly from the bubble jet head, for the properties of
the above-described liquid, it is preferable that its
viscosity be in the range of 1 to 15 cps and its surface
tension be 30 dyn/cm or larger. Also, if the viscosity is in
30 the range of 1 to 5 cps and the surface tension is in the
range of 30 to 50 dyn/cm, the position in which the droplet is
spotted (spot position) on the solid phase is extremely
accurate.

In addition, if the stability of nucleic acid during
35 discharge or the like is taken into consideration, a single-
stranded nucleic acid or double-stranded nucleic acid of, for

5 example, 2 to 5000 mer, particularly 2 to 10000 mer, is preferably contained in the solution. For example, c-DNA chips are preferably contained at the concentration of 0.05 to 500 μM , particularly 2 to 50 μM .

The composition of liquid is not particularly limited, as
10 long as the liquid has no substantial influence on the nucleic acid probe when it is mixed with the nucleic acid probe and when it is discharged from the ink jet. The liquid can be normally discharged to the solid phase using the ink jet. Preferable are liquids including glycerin, urea, thiodiglycol
15 or ethylene glycol, isopropyl alcohol, and acetyl alcohol expressed by the following formula:



In the above formula, R^1 , R^2 , R^3 and R^4 represent alkyl groups, specifically linear or branched alkyl groups having 1
20 to 4 carbon atoms, m and n represent 0 or positive integer numbers, respectively, and satisfy $1 \leq m + n \leq 30$.

Further, specifically, a liquid containing 5 to 10% by weight (wt%) of urea, 5 to 10 wt% of glycerin, 5 to 10 wt% of thiodiglycol, and 0.02 to 5 wt%, more preferably 0.5 to 1 wt%
25 of acetylene alcohol, is suitably used.

Examples

The present invention will be described in detail below using Examples. Furthermore, the Examples shown herein

5 represent most suitable embodiments of the present invention,
and the invention should not be limited by these Examples.

Example 1

A glass substrate with black matrices for specimen
matrices for analyzing sequences of p 53 genes on a specimen
10 matrix substrate partitioned by patterns is prepared.

1. Preparation of a black matrix introduction substrate
coated with polylysine.

A glass substrate (60 mm x 50 mm) made of synthetic
quartz is subjected to supersonic cleaning using 2% sodium
15 hydrate solution, and is then subjected to UV ozone processing
to clean the surface. Then, a polylysine solution
(manufactured by Sigma Co., Ltd.) is applied to the entire
surface with a spin coater. In addition, a DEEP-UV resist
(negative type resist for black matrices) (BK-739P
20 manufactured by Nippon Steel Chemical Co., Ltd.) is applied
thereto with the spin coater so that the thickness after
curing is 5 μm , and this substrate is heated for curing at
80°C for 5 minutes with a hotplate. Using a DEEP-UV aligner,
a region of 1 cm x 1 cm is proximately exposed to light using
25 a patterned mask, so that the distance (X) between adjacent
wells in FIG. 1 is 100 μm and the form of the well is a 1 mm x
1 mm square, and then development is carried out with a
developing inorganic alkaline solution using a spin drier, and
the developing solution is washed out completely with purified
30 water.

Then, the substrate is briefly dried using the spin
drier, and is thereafter heated at 180°C for 30 minutes in a
clean oven to have the resist fully cured to obtain a
substrate in which 400 wells are at a predetermined
35 arrangement and adjacent wells are separated from each other

5 by the black matrix. Furthermore, the volume of each well is calculated as 5 μ l if the thickness of the liquid is 5 μ m.

2. Fixing Specimen DNA

(1) Preparation of cDNA libraries

The p 53 gene is obtained by a PCR reaction from 64 types
10 of cDNA libraries obtained from tumor tissues.

That is, RNA samples were obtained from each tissue collected with biopsies using Catrimox-14 (Biotechnology Co., Ltd.). Based on this sample solution, First-Strand cDNA Synthesis Kit (manufactured by Life Sciences Co., Ltd) is used
15 to obtain cDNA libraries.

(2) Amplification of p53 genes having T3 binding sites by a PCR method

Based on the cDNA library, "Human p53 Amplimer set" manufactured by CLONTECH Co., Ltd. is used to carry out the
20 PCR reaction.

As a PCR reaction solution, "one shot LA PCR Mix" (Takara Shuzo Co., Ltd.) was used. The composition of the PCR reaction solution is as follows:

| | |
|------------------------|----------------|
| One shot LA PCR Mix | 25 μ l |
| 5' primer (20 μ M) | 1 |
| 3' primer (20 μ M) | 1 |
| cDNA library solution | 1 |
| DW | 22/50 μ l. |

The PCR cycle is such that after thermal denaturation at
25 95°C for 5 minutes, cycles at 95°C for 30 seconds, at 55°C for 30 seconds and at 72°C for 60 seconds are conducted 29 times. Finally, the solution is left for a reaction at 72°C for 5 minutes and is then stored at 4°C.

After the reaction, gel electrophoresis is performed to
30 confirm a product existing in the region of molecular weight

5 of about 300 mer, and purification is carried out with
MicroSpin Column S200 (Pharmacia) to obtain p 53 genes (p 53
DNA).

(3) Synthesis of single-stranded p 53 DNA

Using as a matrix the DNA obtained in the above (2), a
10 single-stranded marked DNA is obtained by the PCR reaction
using 5' primer (Takara Shuzo Co., Ltd.). The composition of
the reaction solution comprises

| | |
|------------------------|--------------------|
| One shot LA PCR Mix | 25 μ l |
| 5' primer (20 μ M) | 1 |
| P 53 DNA | 1 |
| DW | 22/50 μ l, and |

the reaction cycle is such that cycles at 96°C for 30 seconds,
at 60°C for 15 seconds and at 60°C for 4 minutes are repeated
15 24 times, and finally, the solution is stored at 4°C.
Thereafter, it is purified with MicroSpin Column S200.

(4) Fixing p 53 cDNA

5 μ l of the single-stranded DNA obtained in the above (3)
is injected under a microscope into each well of the
20 polylysine-coated substrate with black matrices prepared in
the above (1), and is fixed through electrostatic coupling.

3. Analysis of variation of p 53 genes with
oligonucleotide probes

The 64 DNAs were selected, focusing the attention on the
25 248th and 249th amino acid sequences of the p 53 gene being a
tumor inhibitor gene. That is, it is known that a case of
frequent variation in the base sequence of CGGAGG is the case
where the first C is changed to T, the second A is changed to
G, and the third G of the sequence corresponding to the 249th
30 amino acid is changed to T. Thus, the 64 probes are designed,

5 focusing the attention on the base sequence at these three points.

That is, it is a structure in which the total length of the probe is 18 mer, and six bases including this variation are located at the center thereof, and the bases are
10 sandwiched between common sequences. A common sequence corresponds to the range from the five prime-end to the ATGAAC, and the subsequent portion including variation corresponds to the NNGAGN and a further subsequent common portion corresponds to the CCCATC, resulting in a final
15 sequence of 5'ATGAACNNGAGNCCCATC3' (SEQ ID NO:65). Here, the portion expressed by N corresponds to the A, G, C, and T that are four types of nucleic acid bases. The probe DNA has a sequence complementary to the sequence to be detected (the above-described sequence), and thus the sequence thereof is
20 5'GATGGGNCTCNNGTTCAT3' (SEQ ID NO:66). Rhodamine is coupled to the five prime-end of each probe sequence to mark the probe. Specific base sequences of these 64 types of marked DNA probes are shown in the following Table 1.

5

Table 1

| SEQ ID NO. | Sequence | SEQ ID NO. | Sequence |
|------------|---------------------|------------|---------------------|
| 1 | GATGGGACTCAAGTTCAT | 33 | GATGGGCCTCAAGTTCAT |
| 2 | GATGGGACTCAGGTTCAT | 34 | GATGGGGCTCAGGTTCAT |
| 3 | GATGGGACTCACGTTCAT | 35 | GATGGGCCTCACGTTCAT |
| 4 | GATGGGACTCATGTTCAT | 36 | GATGGGCCTCATGTTCAT |
| 5 | GATGGGACTCGAGTTCAT | 37 | GATGGGCCTCGAGTTCAT |
| 6 | GATGGGACTCGGGTTCAT | 38 | GATGGGCCTCGGGTTCAT |
| 7 | GATGGGACTCGCGTTCAT | 39 | GATGGGCCTCGCGTTCAT |
| 8 | GATGGGACTCGTGTTTCAT | 40 | GATGGGCCTCGTGTTTCAT |
| 9 | GATGGGACTCCAGTTCAT | 41 | GATGGGCCTCCAGTTCAT |
| 10 | GATGGGACTCCGGTTCAT | 42 | GATGGGCCTCCGGTTCAT |
| 11 | GATGGGACTCCCGTTCAT | 43 | GATGGGCCTCCCGTTCAT |
| 12 | GATGGGACTCCTGTTCAT | 44 | GATGGGCCTCCTGTTCAT |
| 13 | GATGGGACTCTAGTTCAT | 45 | GATGGGCCTCTAGTTCAT |
| 14 | GATGGGACTCTGGTTCAT | 46 | GATGGGCCTCTGGTTCAT |
| 15 | GATGGGACTCTCGTTCAT | 47 | GATGGGCCTCTCGTTCAT |
| 16 | GATGGGACTCTTGTTTCAT | 48 | GATGGGCCTCTTGTTTCAT |
| 17 | GATGGGGCTCAAGTTCAT | 49 | GATGGGTCTCAAGTTCAT |
| 18 | GATGGGGCTCAGGTTCAT | 50 | GATGGGTCTCAGGTTCAT |
| 19 | GATGGGGCTCACGTTCAT | 51 | GATGGGTCTCACGTTCAT |
| 20 | GATGGGGCTCATGTTCAT | 52 | GATGGGTCTCATGTTCAT |
| 21 | GATGGGGCTCGAGTTCAT | 53 | GATGGGTCTCGAGTTCAT |
| 22 | GATGGGGCTCGGGTTCAT | 54 | GATGGGTCTCGGGTTCAT |
| 23 | GATGGGGCTCGCGTTCAT | 55 | GATGGGTCTCGCGTTCAT |
| 24 | GATGGGGCTCGTGTTTCAT | 56 | GATGGGTCTCGTGTTTCAT |
| 25 | GATGGGGCTCCAGTTCAT | 57 | GATGGGTCTCCAGTTCAT |
| 26 | GATGGGGCTCCGGTTCAT | 58 | GATGGGTCTCCGGTTCAT |
| 27 | GATGGGGCTCCCGTTCAT | 59 | GATGGGTCTCCCGTTCAT |
| 28 | GATGGGGCTCCTGTTCAT | 60 | GATGGGTCTCCTGTTCAT |
| 29 | GATGGGGCTCTAGTTCAT | 61 | GATGGGTCTCTAGTTCAT |
| 30 | GATGGGGCTCTGGTTCAT | 62 | GATGGGTCTCTGGTTCAT |
| 31 | GATGGGGCTCTCGTTCAT | 63 | GATGGGTCTCTCGTTCAT |
| 32 | GATGGGGCTCTTGTTTCAT | 64 | GATGGGTCTCTTGTTTCAT |

5 Then, for each of the 64 types of marked probe DNAs, a 8
µM solution containing glycerin, urea and thiodiglycol at the
final concentration of 7.5%, and acetylenol EH at the final
concentration of 1% is prepared. A different probe solution
is charged by 100 µl in each of the six nozzles of BJ Printer
10 Head BC 62 (manufactured by Canon Inc.). An arrangement is
made so that six DNAs can be discharged for each head, and two
heads are used to discharge 12 DNAs at a time, and the heads
are exchanged 6 times to discharge DNAs so that each spot of
64 DNA is formed independently. In this manner, a total of 64
15 probes are discharged in the form of the 8 x 8 array in each
well of a black matrix coated with polylysine.

FIG. 5 shows an arrangement on each black matrix of 64
DNA probes that are discharged. In this case, 64 DNA probes
are spotted in one matrix.

20 Thereafter, this substrate in which each probe is spotted
is left in a humidifier chamber set at 40°C to carry out a
hybridization reaction.

Thereafter, the substrate is cleaned with a 10 mM
phosphate buffer containing 100 mM NaCl to remove DNA probes
25 that have not been engaged in the formation of the hybrid
substance.

DNA arrays after the hybridization reaction are observed
using an inverted fluorescence microscope equipped with a
filter set suitably for rhodamine.

30 If the gene as a specimen has normal base sequences,
spots of highest fluorescence intensity should be observed in
the gene at the location of the relative 42nd DNA probe. It
can be considered that those are derived from the hybrid of
the p 53 gene having normal sequences exposed to the probe DNA
35 and PCR. In a varied gene, detectable spots are observed at

5 the location other than the 42nd location, and a varied
sequence can be identified from the DNA probe supplied to the
location.

Example 2

Evaluation of Existence/Non-existence of Carcinogenic
10 Genes Using mRNA

1. Extraction of mRNA

"QuickPrep Micro mRNA Purification Kit" (manufactured by
Amersham Pharmacia Biotech co., Ltd.) is used to extract mRNA
from tumor tissues collected with the biopsy. This mRNA is
15 bound to a polylysine substrate with black matrices, as in the
case of Example 1.

2. Examination of existence/non-existence of
carcinogenic genes and the type thereof with various kinds of
carcinogenic gene probe arrays

20 Sets of cloned oncogenes (18 types, manufactured by
Takara Shuzo Co., Ltd.) are purchased. Then, "LabelITnon-R1
Labeling Kits" are used to perform rhodamine marking.

18 types of marked oncogene probes are spotted as an
arrangement of 4 x 5 on the above-described substrate with
25 mRNA bound thereto, using a microarray preparing apparatus
(pin system) manufactured by Cartesian Technologies Co., Ltd.

Further, a hybridization reaction is carried out as in
Example 1.

The type of oncogenes existing in the mRNA section
30 extracted from each tissue can be known.

At this time, sufficient detection can be performed with
one type of marks irrespective of the types of oncogenes
present.

The second aspect of the invention is described more
35 specifically below with reference to the Examples.

5 Example 3

 An example of procedures for preparing a substrate with oligonucleotide bound thereto will be described below. In this embodiment, a detection substrate with the oligonucleotide bound to a region of 2 mm square on a glass
10 substrate was prepared in accordance with the procedure described below.

 1. Cleaning of the substrate

 A glass substrate of 1-inch square was placed on a rack and soaked in a detergent for ultrasonic cleaning.
15 Thereafter, it was subjected to ultrasonic cleaning in the above-described detergent for 20 minutes, followed by removing the detergent by rinsing. Also, it was rinsed with distilled water, followed by further performing ultrasonication for 20 minutes in a container containing distilled water.

20 Then, this glass substrate was soaked for 10 minutes in 1N sodium hydrate solution heated in advance. After it was taken out from the solution, the 1N sodium hydrate solution adhered to the surface was washed out with water. Thereafter, cleaning with distilled water was continued.

25 2. Surface treatment

 The above-described cleaned glass substrate was soaked in an aqueous solution of 1% silane coupling agent (manufactured by Shin-Etsu Chemical Co., Ltd., Trade name: KBM 603) at room temperature for 20 minutes, followed by spraying nitrogen gas
30 on both sides of the substrate to drive off water for drying. The substrate was baked for one hour by using an oven heated to 120°C to complete the treatment of the surface of the glass substrate with a silane coupling agent.

 On the other hand, 2.7 mg of EMCS (N-(6-
35 Maleimidocaproyloxy) succinimide: manufactured by Dojin Co., Ltd.) was weighed, and was dissolved in a solution of

5 DMSO/ethanol (1:1) (final concentration of 0.3 mg/ml). The
glass substrate subjected to the treatment with a silane
coupling agent was soaked in this EMCS solution for two hours
to carry out the reaction between the amino group of the
silane coupling agent covering the surface of the substrate
10 and the succinimide group in the EMCS solution. In
association with this reaction, the substrate is covered with
EMCS through the silane coupling agent. In the obtained glass
surface, a maleimide group derived from the EMCS is present on
the surface. The glass substrate taken out after the reaction
15 with the EMCS solution is cleaned with distilled water, and is
thereafter dried with nitrogen gas. This glass substrate
subjected to the surface treatment for introducing a maleimide
group will be used for a binding reaction with DNA described
below.

20 3. Synthesis of DNA for fixing glass substrates

An oligonucleotide having a base sequence of the
following Sequence 1 (SEQ ID NO:42) is chemically synthesized
for fixing on the glass substrate. This sequence 1 is a 18
mer sequence including in its central part a base sequence
25 with a base length of 6 to code 248th and 249th amino acids in
an amino acid sequence of a gene product (peptide chain) that
is coded by the p 53 gene known as a tumor suppressor gene.
Also, A SH group is introduced in its 5' end for fixation on
the glass substrate.

30 Sequence 1 5' HS-GATGGGCCTCCGGTTCAT3' (SEQ ID NO:42)

The SH group is introduced by using a commercially
available reagent Thiol-Modifier (manufactured by GlenResearch
Co., Ltd.) on a DNA automatic synthesizing apparatus.
Subsequently, normal deprotection was carried out to recover
35 DNA. The DNA was purified by high-speed liquid
chromatography, and was then used in the following processes.

5 4. Discharging of DNA using a BJ printer head and
binding thereof to a substrate

 The above-described synthetic oligonucleotide (DNA) was
dissolved in water, and the solution was diluted to a
concentration of 8 μ M using SG Clear (a solution containing
10 7.5% of glycerin, 7.5% of urea, 7.5% of thiodiglycol and 1% of
acetylenol EH).

 100 μ l of this oligonucleotide solution was charged into
the nozzle of BJ printer head BC 62 (manufactured by Canon
Inc.) with the nozzle modified so that it is suitable for a
15 small amount of samples (discharged amount). This modified
printer head was set in a plotting apparatus to perform
printing over the surface of the glass substrate as an area of
"solid print" of 2 mm square with the oligonucleotide
solution. Furthermore, the modified printer head that was
20 used is used for bubble jet type ink jet printing and enables
printing to be performed at a resolution of 360 x 720 dpi.

 Thereafter, the glass substrate coated with the
oligonucleotide solution was left in a humidifier chamber for
30 minutes to carry out a reaction between the maleimide group
25 on the surface of the substrate and the thiol group (HS-) of
the oligonucleotide. Thereafter, the unreacted
oligonucleotide was removed. The prepared substrate for
detection has the synthetic DNA (oligonucleotide) of the
above-described Sequence 1 bound to a predetermined 2 mm
30 square section on the glass substrate via a covalent bond.

Example 4

 Supply of cDNA solution to the Surface of the Substrate
with Oligonucleotide Bound Thereto and Hybridization Reaction

 From various kinds of cDNA libraries obtained from tumor
35 tissues, p 53 gene fragments were PCR-amplified, and then only

5 one type of side chains was reamplified using primers marked
in advance to prepare marked single-stranded cDNA for use as
test samples. The hybridization reaction was carried out
between this marked single stranded DNA derived from the p 53
gene and the DNA probe bound on the detection substrate
10 prepared in Example 3.

1. Preparation of test samples

From 64 types of cDNA libraries obtained from tumor
tissues, p 53 gene fragments were obtained by the PCR
reaction.

15 Specifically, first, all RNA samples were
separated/collected from respective tissues collected via a
biopsy, using Catrimox-14 (Biotechnology Co., Ltd.). On the
basis of all the RNA sample solutions, a c-DNA library was
prepared using First-Strand cDNA Synthesis Kit (manufactured
20 by Life Science Co., Ltd.). A primer for amplifying p 53
genes was added to this cDNA library to amplify P 53 gene
fragments. With this PCR amplification product as a template,
the marked five-side primer was used to carry out the PCR
reaction (DNA synthetic reaction) to amplify only one type of
25 side chains. By this amplification, marked single stranded
DNA derived from the p 53 gene can be prepared.

(1) Amplification of p 53 gene fragments having a T3
binding site in the terminal by the PCR method

For using a primer for auto sequencers (Takara Shuzo Co.,
30 Ltd) using T3 promoters as the above-described marked primer,
a primer having a T3 site in the terminal and having coupled
to its downstream a base sequence allowing the p 53 gene part
to be amplified was first synthesized. The PCR reaction was
carried out using this primer to obtain a PCR amplification
35 product having a T3 promoter site coupled to the p 53 gene
part.

5 In this example, for the five prime-end primer for amplifying p 53 genes, the primer with a base sequence having a T3 promoter site coupled to its five side (T3-P53), was prepared. The base sequence is shown below.

5'

10 AATTAACCCTCACTAAAGGGAACCTGAGGTTGGCTCTGACTGTACCACCATCC3' (SEQ ID NO:67)

In the sequence, the underlined part on the side of five prime-end represents a T3 polymerase binding site. On the other hand, for a three prime-end primer for amplification, a
15 three prime-end primer attached in a commercially available amplification kit, "Human p 53 Amplimer Set" of CLONTECH Co., Ltd., was used. For a PCR reactive solution, "one shot LA PCR Mix" (Takara Shuzo Co., Ltd.) was used.

The solution composition in the PCR reaction has:

| | |
|----------------------------|------------------------|
| one shot LA PCR Mix | 25 μ l |
| T3-P53 primer (20 μ M) | 1 μ l |
| 3' primer (20 μ M) | 1 μ l |
| cDNA library solution | 1 μ l |
| DW | 22 μ l/50 μ l. |

20 For the PCR cycle, the cycles were conducted at 95°C for 30 seconds, at 55°C for 30 seconds and at 72°C for 60 seconds at 29 times after thermal denaturation at 95°C for 5 minutes, and finally keeping the solution at 72°C for five minutes. The reactant was stored at 4°C on a temporary basis after it
25 was cooled.

After the reaction, gel electrophoresis was carried out to confirm a PCR product present in the region of molecular weight of about 300 mer. This PCR product was purified with Micro Spin Column 5200 (Pharmacia) to obtain p 53 gene
30 fragments to which the T3 primer can be coupled (T3-linked p 53 DNA).

5 (2) Synthesis of marked single-stranded DNA using
labeled T3 primers (Rho-T3)

With the p 53 gene fragment obtained in (1) as a matrix,
single stranded marked DNA was obtained with the PCR reaction,
using a Rho-T3 primer (Takara Shuzo Co., Ltd.). The

10 composition of the reactive solution had:

| | |
|---------------------------|------------------------|
| one shot LA PCR Mix | 25 μ l |
| Rho-T3primer (10 μ M) | 1 μ l |
| T3-linked p 53 DNA | 1 μ l |
| DW | 23 μ l/50 μ l. |

For the reaction cycle, the cycles were conducted at 96°C
for 30 seconds, at 50°C for 15 seconds and at 60°C for 4
minutes 24 times. The reactant was stored at 4°C on a
temporary basis after it was cooled. It was purified with
15 Micro Spin Column S200. Thereafter, gel electrophoresis was
carried out to confirm desired rhodamine labeled single-
stranded DNA synthesized through the PCR reaction.

2. Supply of test sample solution

Sodium chloride was added in the test sample obtained in
20 the above-described process, namely the solution of rhodamine-
marked single-stranded DNA derived from the p 53 gene, so that
the final concentration of the solution was 1M. The solution
of rhodamine-marked single-stranded DNA derived from the p 53
gene, which had been prepared from 64 types of c DNA

25 libraries, was injected into each well of a 96-hole microtiter
plate. These solutions of rhodamine-marked single-stranded
DNA were spotted in an 8 x 8 arrangement onto the detection
glass substrate with the DNA probe of Sequence 1 obtained in
Example 3 in the form of 2 mm square, using a microarray
30 preparing apparatus (pin system) manufactured by Cartesian
Technologies. The diameter of each spot was 100 μ m.

5 3. Hybridization reaction

 This detection substrate with a total of 64 types of rhodamine-marked single-stranded DNA solutions being sample specimens spotted thereon was left in a humidifier chamber set at 40°C to carry out a hybridization reaction for 3 hours.

10 Thereafter, the detection substrate was washed with a 10 mM phosphate buffer containing 100 mM NaCl to remove test samples that had not been engaged in the formation of hybrid substances.

 After the hybridization reaction, the test sample spotted
15 in the form of a two-dimensional 8 x 8 array was observed using an inverted fluorescence microscope equipped with a filter set for excitation light and fluorescence suitable for fluorescence marked rhodamine. For most of the spots, red fluorescence derived from fluorescence marked rhodamine in
20 association with the formation of hybrid substances was observed. However, fluorescence intensity was weak for six spots and no fluorescence was observed for one spot.

 Due to this, it can be considered that since in the p 53 gene derived from corresponding six types of tumor cells
25 variation occurs somewhere in the base sequence corresponding to the 248th and 249th of the amino acid sequence of the p 53 gene product (p 53 protein), the amount of formed hybrid substances is small due to its mismatch. In that connection, the fluorescence intensity from the fluorescence mark is weak.
30 For the test sample in which fluorescence was not observed, it can be considered that because hybrid substances in p 53 cDNA fragments did not form, a deficiency occurs in the base sequence to code the above-described 248th and 249th amino acid sequence. Consequently, hybrid substances could not be
35 formed.

5 Example 5

Preparation of Array form Spots of Test Samples on the
Probe Matrix Detection Substrate with Multiple
Oligonucleotides Fixed Thereon

1. Preparation of 64 probe matrices

10 Processing was performed as in Example 3 to prepare a
glass substrate having a maleimide group. 64 DNAs with the
base sequences as shown in Table 2 were printed (applied)
thereon in the area of 2 mm square, respectively, using a
bubble jet printer head similar to that in Example 3 to
15 prepare a detection substrate on which sections with 64 types
of prove DNAs fixed therein were arranged in a matrix form.

Focusing the attention on the 248th and 249th amino acids
of the amino acid sequence of the gene product (p 53 protein)
of the p 53 gene being a tumor suppressor gene, 64 DNAs with
20 base sequences as shown in Table 1 were selected on the basis
of the base sequence to code these two amino acids so that a
sequence with various kinds of base variations added thereto
was obtained. Specifically, it is known that a case of
frequent variation in the base sequence CGGAGG providing a
25 base is the case where the first C of the CGG to code the
248th amino acid is changed to T, the second A is changed to
G, and the third G of the AGG to code the 248th amino acid is
changed to T. Thus, 64 probes were designed to provide
sequences capable of being bound to base sequences with these
30 bases at three positions varied in various ways.

Specifically, it was a structure in which the total
length of the probe was 18 mer, six bases including this
variation were located in the center thereof, and common base
sequences with base lengths of 6 were placed before and after
35 the six bases. More specifically, the structure has a common
sequence of ATGAAC from the side of the five prime-end, the

5 base sequence of NNGAGN as a portion including the variation,
and a common sequence of CCCATC on the side of three prime-
end.

It was a base sequence complimentary to the sequence of
5'ATGAACNNGAGNCCCATC3'(SEQ ID NO:65). That is, it was a probe
10 expressed by 5'GATGGGNCTCNGTTCAT3' (SEQ ID NO:66).
Furthermore, since it is a DNA probe, the portion denoted by N
in the above-described base sequence refers to any one of A,
G, C and T that are four DNA nucleic acid bases.

5

Table 2

| | | | |
|----|---------------------------|----|---------------------------|
| 1 | 5'-GATGGGACTCAAGTTCAT-3' | 33 | 5'-GATGGGCCTCAAGTTCAT-3' |
| 2 | 5'-GATGGGACTCAGGTTCAT-3' | 34 | 5'-GATGGGCCTCAGGTTCAT-3' |
| 3 | 5'-GATGGGACTCACGTTCAT-3' | 35 | 5'-GATGGGCCTCACGTTCAT-3' |
| 4 | 5'-GATGGGACTCATGTTCAT-3' | 36 | 5'-GATGGGCCTCATGTTCAT-3' |
| 5 | 5'-GATGGGACTCGAGTTCAT-3' | 37 | 5'-GATGGGCCTCGAGTTCAT-3' |
| 6 | 5'-GATGGGACTCGGGTTCAT-3' | 38 | 5'-GATGGGCCTCGGGTTCAT-3' |
| 7 | 5'-GATGGGACTCGCGTTCAT-3' | 39 | 5'-GATGGGCCTCGCGTTCAT-3' |
| 8 | 5'-GATGGGACTCGTGTTTCAT-3' | 40 | 5'-GATGGGCCTCGTGTTTCAT-3' |
| 9 | 5'-GATGGGACTCCAGTTCAT-3' | 41 | 5'-GATGGGCCTCCAGTTCAT-3' |
| 10 | 5'-GATGGGACTCCGGTTCAT-3' | 42 | 5'-GATGGGCCTCCGGTTCAT-3' |
| 11 | 5'-GATGGGACTCCCGTTCAT-3' | 43 | 5'-GATGGGCCTCCCGTTCAT-3' |
| 12 | 5'-GATGGGACTCCTGTTCAT-3' | 44 | 5'-GATGGGCCTCCTGTTCAT-3' |
| 13 | 5'-GATGGGACTCTAGTTCAT-3' | 45 | 5'-GATGGGCCTCTAGTTCAT-3' |
| 14 | 5'-GATGGGACTCTGGTTCAT-3' | 46 | 5'-GATGGGCCTCTGGTTCAT-3' |
| 15 | 5'-GATGGGACTCTCGTTCAT-3' | 47 | 5'-GATGGGCCTCTCGTTCAT-3' |
| 16 | 5'-GATGGGACTCTTGTTTCAT-3' | 48 | 5'-GATGGGCCTCTTGTTTCAT-3' |
| 17 | 5'-GATGGGGCTCAAGTTCAT-3' | 49 | 5'-GATGGGTCTCAAGTTCAT-3' |
| 18 | 5'-GATGGGGCTCAGGTTCAT-3' | 50 | 5'-GATGGGTCTCAGGTTCAT-3' |
| 19 | 5'-GATGGGGCTCACGTTCAT-3' | 51 | 5'-GATGGGTCTCACGTTCAT-3' |
| 20 | 5'-GATGGGGCTCATGTTCAT-3' | 52 | 5'-GATGGGTCTCATGTTCAT-3' |
| 21 | 5'-GATGGGGCTCGAGTTCAT-3' | 53 | 5'-GATGGGTCTCGAGTTCAT-3' |
| 22 | 5'-GATGGGGCTCGGGTTCAT-3' | 54 | 5'-GATGGGTCTCGGGTTCAT-3' |
| 23 | 5'-GATGGGGCTCGCGTTCAT-3' | 55 | 5'-GATGGGTCTCGCGTTCAT-3' |
| 24 | 5'-GATGGGGCTCGTGTTTCAT-3' | 56 | 5'-GATGGGTCTCGTGTTTCAT-3' |
| 25 | 5'-GATGGGGCTCCAGTTCAT-3' | 57 | 5'-GATGGGTCTCCAGTTCAT-3' |
| 26 | 5'-GATGGGGCTCCGGTTCAT-3' | 58 | 5'-GATGGGTCTCCGGTTCAT-3' |
| 27 | 5'-GATGGGGCTCCCGTTCAT-3' | 59 | 5'-GATGGGTCTCCCGTTCAT-3' |
| 28 | 5'-GATGGGGCTCCTGTTCAT-3' | 60 | 5'-GATGGGTCTCCTGTTCAT-3' |
| 29 | 5'-GATGGGGCTCTAGTTCAT-3' | 61 | 5'-GATGGGTCTCTAGTTCAT-3' |
| 30 | 5'-GATGGGGCTCTGGTTCAT-3' | 62 | 5'-GATGGGTCTCTGGTTCAT-3' |
| 31 | 5'-GATGGGGCTCTCGTTCAT-3' | 63 | 5'-GATGGGTCTCTCGTTCAT-3' |
| 32 | 5'-GATGGGGCTCTTGTTTCAT-3' | 64 | 5'-GATGGGTCTCTTGTTTCAT-3' |

Then, for each of the 64 types of labeled probe DNAs, an 8 μ M solution containing glycerin, urea and thiodiglycol at the final concentration of 7.5%, respectively, and acetylenol EH at the final concentration of 1% was prepared. As in

5 Example 4, using BJ Printer Head BC 62 (manufactured by Canon Inc), a different DNA probe solution was charged by 100 μ l in each of the six nozzles of the printer head. Using a plurality of such printer heads, a detection substrate with total 64 DNA probes applied to and fixed in each section of 2
10 mm square in the form of a "solid print" and arranged in a matrix form (8 x 8) was prepared. A schematic layout of the 64 DNA probes arranged in a matrix form (8 x 8) on the detection substrate is shown in FIG. 7.

2. Preparation of array spots of test samples.

15 As in the case of Example 4, 64 types of labeled cDNAs were spotted in the form of the two-dimensional 8 x 8 array on each region of 2 mm square for fixing probes. Specifically, as schematically shown in FIG. 8, a pin system array preparing apparatus was used to form spots in the form of the two-
20 dimensional 8 x 8 array on the sections arranged in a matrix form (8 x 8) in which each DNA probe was fixed.

3. Hybridization reaction

A hybridization reaction was carried out using conditions and procedures similar to those in Example 4. The result
25 thereof is shown in FIG. 9. In the arrangement shown in FIG. 7, with respect to spots on probes corresponding to the base sequence of the 42nd normal gene, fluorescence intensity was weak for six spots as in Example 4. Also, no fluorescence was observed for one spot. In addition thereto, it was observed
30 that fluorescence was emitted from the spot at three points in the tenth probe region, at two points in the 41st probe region, and at one point in the 46th probe region, respectively.

Spot positions in which fluorescence in association with
35 the formation of hybrid substances was observed in the prove

5 region having these base sequences including variations
corresponded to spot positions of weak fluorescence intensity
in the probe region having the above-described 42nd original
base sequence. Thus, if the base sequences of the probes are
10 compared between both regions, the base sequence of the tenth
probe is ACTCCG, the base sequence of the 41st probe is the
CCTCCA, and the base sequence of the 46th probe is CCTCTG with
respect to the original base sequence of CCTCCG of the 42nd
probe. For their complementary sequences, it can be
understood that with respect to CGGAGG in the 42nd probe, the
15 CGGAGT and G were changed to T in the tenth probe, the TGGAGG
and C were changed to T in the 41st probe, and the CAGAGG and
G were changed to A in the 46th probe. That is, it was
confirmed that in test samples, forming hybrid substances with
these tenth, 41st and 46th probes, cDNA fragments contained
20 therein derived from the p53 gene caused one base mismatch
with respect to the 42nd probe due to the above-described
variations.

By this method, existence/non-existence of variations and
types thereof could be detected at the same time for all the
25 64 types of test samples.

Example 6

Preparation of a Substrate for Probe Matrices Partitioned by Patterns

A glass substrate with an epoxy group introduced to the
30 surface and with black matrices for probe matrices was
prepared in accordance with the following procedure.

1. Introduction of an epoxy group to the surface of the
substrate

A glass substrate made of synthetic quartz (50 mm x 50
35 mm) was first subjected to ultrasonic cleaning using a 2%
sodium hydrate solution, and was then subjected to UV ozone

5 processing to clean the surface. A 50% methanol solution
containing 1% of a silane coupling agent (trade name: KBM
403; manufactured by The Shin-Etsu Chemical Co., Ltd.)
containing a silane compound having an epoxy group bonded
thereto (γ -glycidoxypyrpyltrimethoxysilane) was stirred at
10 room temperature for three hours to perform a preliminary
treatment for hydrolyzing the methoxy group in the silane
compound. This solution already subjected to the hydrolysis
treatment was applied to the surface of the above-described
clean substrate with a spin coater, and was heated and dried
15 at 100°C for 5 minutes to form a binding coating of the silane
coupling agent on the surface of the substrate. Through the
formation of this coating, the epoxy group contained in the
silane compound was introduced to the surface of the
substrate.

20 2. Formation of black matrices

Then, A DEEP-UV resist containing carbon black (negative
type resist for black matrices) (trade name: BK-739P;
manufactured by Nippon Steel Chemical Co., Ltd.) was applied
on the surface of the substrate with a spin coater, so that
25 the film thickness after curing was 5 μ m, and it was heated
for curing on a hotplate at 80°C for 5 minutes. By the
proximity exposure using a DEEP-UV aligner, a pattern was
exposed to light using as an exposure mask a mask for
negatives with patterning applied to a region of 10 mm x 10
30 mm, so that the distance X between adjacent wells was 100 μ m
and the outer shape of the well was a 1 mm x 1 mm square.
Then, the development was carried out with an inorganic
aqueous alkaline developer solution using a spin drier, and
the substrate was washed with pure water to completely remove
35 the developer. Then, it was briefly dried using the spin

5 drier, and was thereafter heated in a clean oven at 180°C for
30 minutes to fully cure the resist. As a whole, a substrate
with 400 wells in a predetermined arrangement and black
matrices (resist walls) partitioning adjacent wells was
obtained. Furthermore, the internal volume of each well is
10 calculated as 5 μ l if the thickness of the solution is 5 μ m.
Also, in the surface of the prepared black matrix, the angle
of contact to water was 93 degrees and wettability with water
was significantly low, while in the bottom of the well, the
angle of contact to water was 35 degrees and the wettability
15 with water was high.

3. Fixing probe DNA

64 oligonucleotides of 18 mer with an amino group bound
to the hydroxyl group of the five prime-end through a
phosphate group and hexamethylene were prepared as DNA probes.
20 The 64 probes are same as those prepared in Example 5 with
respect to base sequences, but are different in the sense that
an amino group is introduced in their five prime-end instead
of a thiol group.

5 μ l of solution of these DNA probes was injected into
25 each well under a microscope, and was left in a humidified
chamber to allow the probe to bind to the substrate through
the reaction between the amino group of the five primer-end
and the epoxy group on the substrate.

Example 7

30 Analysis of cDNA derived from the p 53 gene that has been
prepared from mRNA, using the probe matrix substrate
partitioned by the pattern that has been prepared in Example 6
is as follows.

As in the case of Example 4, 64 types of labeled cDNAs
35 were spotted in each probe region of 2 mm square as an

5 arrangement of 8 x 8 spots, as shown in FIG. 8, using a pin system array preparing apparatus.

A hybridization reaction was carried out by a method similar to that in Example 4.

The obtained result was similar to that in Example 5.

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